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<b>(21) International Application Number:</b> PCT/US91/02968 <b>(22) International Filing Date:</b> 29 April 1991 (29.04.91)  <b>(30) Priority data:</b> 518,447                      3 May 1990 (03.05.90)                      US  <b>(60) Parent Application or Grant</b> <b>(63) Related by Continuation</b> US                                      Not furnished (CIP)  <b>(71) Applicants (for all designated States except US):</b> CORNELL RESEARCH FOUNDATION, INC. [US/US]; 20 Thornwood Drive, Ithaca, NY 14850 (US). CALIFORNIA INSTITUTE OF TECHNOLOGY [US/US]; 1301 E. California Boulevard, Pasadena, CA 91125 (US).	<b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only) :</b> BARANY, Francis [US/US]; 1161 York Avenue, New York, NY 10000 (US). ZEBALA, John [US/US]; 420 East 70th Street, New York, NY 10000 (US). NICKERSON, Deborah, A. [US/US]; 975 San Pasqual Street, Pasadena, CA 91106 (US). KAISER, Robert, J., Jr. [US/US]; 897 Monterey Road, Glendale, CA 91206 (US). HOOD, Leroy [US/US]; 1453 East California, Pasadena, CA 91106 (US).  <b>(74) Agent:</b> YAHWAK, George, M.; Yahwak & Associates, 25 Skytop Drive, Trumbull, CT 06611 (US).  <b>(81) Designated States:</b> AT (European patent), BE (European patent), CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), GR (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), US.  <b>Published</b> <i>With international search report.</i>	
<b>(54) Title:</b> A THERMOSTABLE LIGASE MEDIATED DNA AMPLIFICATION SYSTEM FOR THE DETECTION OF GENETIC DISEASES  <b>(57) Abstract</b>  The present invention relates to the cloning of the gene of a thermophilic DNA ligase, from <i>Thermus aquaticus</i> strain HB8, and the use of this ligase for the detection of specific sequences of nucleotides in a variety of nucleic acid samples, and more particularly in those samples containing a DNA sequence characterized by a difference in the nucleic acid sequence from a standard sequence including single nucleic acid base pair changes, deletions, insertions or translocations.		

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## A THERMOSTABLE LIGASE MEDIATED DNA AMPLIFICATION SYSTEM FOR THE DETECTION OF GENETIC DISEASES

More than 2,000 conditions have been identified as single-  
5 gene defects for which the risk of producing affected offspring  
can be mathematically predicted. Among these conditions in man  
include Huntington's chorea, cystic fibrosis, alpha<sub>1</sub> antitrypsin  
deficiency, muscular dystrophy, Hunter's syndrome, Lesch-Nyhan  
syndrome, Down's syndrome, Tay-Sachs disease, hemophilias,  
10 phenylketonuria, thalasemias, and sickle-cell anemia.

Three important techniques have been developed recently  
for directly detecting these single nucleic acid base pair changes,  
deletions, insertions, translocations or other mutations.  
However, two of these techniques cannot be easily automated. In  
15 the first such technique, the presence or absence of the mutation  
in a patient's clinical sample is detected by analysis of a  
restriction digest of the patient's DNA using Southern blotting  
[see Journal of Molecular Biology 98:503 (1975)]. However, the  
Southern blotting technique cannot be used for genetic diseases  
20 where the mutation does not alter a restriction site as, for  
example in alpha<sub>1</sub> antitrypsin deficiency. The second technique is  
by the use of DNA probes which involves the synthesis of an  
oligonucleotide of about 19 base pairs that is complementary to  
the normal DNA sequence around the mutation site. The probe is  
25 labelled and used to distinguish normal from mutant genes by  
raising the stringency of hybridization to a level which the probe

will hybridize stably to the normal gene, but not to the mutant gene with which it has a single base pair mismatch [see Proc. Natl. Acad. Sci. USA 80:278 (1983)]. The original method has been modified by immobilizing the oligonucleotide and probing with a labelled PCR amplified sample. In this modification, the sample is allowed to hybridize to an immobilized oligonucleotide and then washed off by raising the stringency of hybridization as described above [see Proc. Natl. Acad. Sci. USA 86:6230 (1989)]. Other methods have been developed which use fluorescent PCR primers to specifically amplify only one mutation or allele [see Proc. Natl. Acad. Sci. USA 86:9178 (1989)]. This method requires the separation of products from primers by spin columns or gel electrophoresis and hence is not amenable to large scale automation. The third technique utilizes the presence of both diagnostic and contiguous probes under conditions wherein the diagnostic probe remains substantially covalently bound to the contiguous probe only in the case wherein the sample nucleic acid contains the exact target sequence. In addition, the diagnostic oligonucleotide probe may contain a "hook" (for example, a biotinylated oligonucleotide) which is captured (for example, by streptavidin) as a means of increasing the efficiency of the technique, and the contiguous probe may contain a detectable moiety or label [see Science 241:1077 (1988) and U.S. Patent 4,883,750].

Although it is not always necessary, the detection of single base pair mutations in DNA is usually preceded by techniques to

increase or amplify the amount of DNA sample material. A number of techniques exist to perform nucleic acid amplification, among which are: (1) polymerase chain reaction which can amplify DNA a million fold from a single copy in a matter of hours using

5 *Taq* polymerase and running 20 to 30 reaction cycles on a temperature cycling instrument [see *Science* 239:487 (1988), and United States Patents 4,683,195, 4,683,202, and 4,800,159]; (2) self-sustained sequence replication or 3SR can amplify DNA or RNA 10 million fold from a single copy in less than an hour using

10 reverse transcriptase, T7 RNA polymerase, and RNase H under isothermal conditions at 37°C [see *Proc. Natl. Acad. Sci. USA* 87:1874 (1990)]; and (3) Q Beta Replicase can replicate a few thousand RNA molecules containing a special 300bp recognition sequence a billion fold in 30 minutes. Additional techniques are

15 available, and one, the ligase chain reaction, is discussed in the following description of the cloned thermophilic ligase according to the present invention.

In addition to various genetic diseases which may be diagnosed utilizing the present invention, various infectious

20 diseases can be diagnosed by the presence in a clinical sample of a specific DNA sequence characteristic of the causative microorganism. These include bacteria, viruses, and parasites. In such procedures, a relatively small number of pathogenic organisms may be present in a clinical sample from an infected

25 patient and the DNA extracted from these organisms may constitute only a very small fraction of the total DNA in the

sample. However, specific amplification of suspected pathogen-specific sequences prior to immobilization and detection by hybridization of the DNA samples should greatly improve the sensitivity and specificity of traditional procedures. In addition, 5 amplification is particularly useful if such an analysis is to be done on a small sample using nonradioactive detection techniques which may be inherently insensitive, or where radioactive techniques are employed, but where rapid detection is desirable.

Although techniques such as these are available, the search 10 for other techniques for determining single base pair mutations continues. The present invention, that is DNA amplification and/or detection by a ligase detection reaction (LDR) or ligase chain reaction (LCR) utilizing the thermophilic DNA ligase from *Thermus aquaticus* to detect a target DNA sequence is part of that 15 continuing effort.

Although other techniques utilizing *E. coli* or T4 DNA ligase for DNA amplification have been attempted, these have been found to be unacceptable because of a high background "noise" levels (after as few as 10 cycles), a condition which does not exist in 20 the ligase chain reaction according to the present invention.

DNA amplification and/or detection has also been attempted utilizing specific ligases. For example, a ligase amplification reaction has been reported [see Gene 76:245 (1989)] that can amplify DNA starting with 500,000 copies in 95 hours, using 75 25 cycles and replenishing the T4 DNA ligase used after each cycle. However, this reported technique is slow and requires the

addition of fresh T4 ligase at each step, both of which requirements make this reported technique unacceptable for automation. The ligase chain reaction according to the present invention allows for amplification of DNA from 200 copies in 3  
5 hours using 30 cycles and does not require the addition of ligase following each cycle.

Throughout the following description of the present invention, terminology specific to the technology field will be used. In order to avoid any misunderstandings as to what is being  
10 referenced, and to provide the reader with a clear understanding of what is being described, the following definitions will be used:

"Amplification" refers to the increase in the number of copies of a particular nucleic acid fragment resulting either from an enzymatic chain reaction (such as a polymerase chain reaction,  
15 a ligase chain reaction, or a self-sustained sequence replication). or from the replication of the vector into which it has been cloned.

"Blunt end ligation" refers to the covalent linkage of two ends of DNA that are completely flush, i.e. have no cohesive end  
20 overhangs.

"Cell", "cell line", and "cell culture" may be used interchangeably and all such designations include progeny. Thus, the words "transformants" or "transformed cells" includes the primary subject cell and cultures derived therefrom without  
25 regard for the number of transfers. It is also understood that all progeny may not be precisely identical in DNA content due to

deliberate or inadvertent mutations. However, all mutant progeny having the same functionality as screened for in the originally transformed cell are included.

"Clone" refers to a group of genetically identical molecules, cells or organisms asexually descended from a common ancestor. "Cloning" is the process of propagating such identical molecules, cells or organisms. Recombinant DNA techniques make it possible to clone individual genes; this is referred to as "molecular cloning".

10 "Covalently attaching" refers to forming a covalent chemical bond between two substances.

"Cycle" refers to a single melting and cooling of DNA. For example, at very high temperatures such as 94°C, virtually all double stranded DNA (independent of length) unwinds and melts. If one cools the temperature (to 45-65°C) in the presence of complementary oligonucleotides, they can hybridize to the correct sequences of the unwound melted DNA. DNA that has been melted and cooled in the presence of complementary oligonucleotides is now a substrate for the DNA ligase reaction. See "T<sub>m</sub>".

20 "Diagnostic portion" refers to that portion of the target sequence which contains the nucleotide change, the presence or absence of which is to be detected. "Contiguous portion" refers to a sequence of DNA which is a continuation of the nucleotide sequence of that portion of the sequence chosen as diagnostic.

25 The continuation can be in either direction.



It will be recognized, based on the following description, that the precise position of the selected oligonucleotide containing the diagnostic portion is arbitrary, except that it must contain the nucleotide(s) which differentiate the presence or absence of the target sequence at one of its ends. Thus, the oligonucleotide containing the contiguous portion continues the sequence of this arbitrarily chosen oligonucleotide containing the diagnostic portion such that the diagnostic nucleotide(s) is at the junction of the two oligonucleotides.

10 "Endonuclease" refers to an enzyme (e.g., restriction endonuclease, DNase I) that cuts DNA at sites within the molecule.

"Expression system" refers to DNA sequences containing a desired coding sequence and control sequence in operable linkage in such a manner that hosts transformed with these sequences are capable of producing the encoded proteins. In order to effect transformation, the expression system may be included on a vector, or the transformed vector DNA may also be integrated into the host chromosome.

20 "Gene" refers to a DNA sequence which encodes a recoverable bioactive polypeptide or precursor. The polypeptide can be encoded by a full-length gene sequence or any portion of the coding sequence so long as the enzymatic activity is retained.

"Gene library" or "library" refers to a collection of randomly-cloned fragments that encompass substantially the

entire genome of a given species. This is also referred to as a clone bank or shotgun collection.

"Genome" refers to the entire DNA of an organism.

"Hook" refers to a modification of a probe that enables the user to rapidly and conveniently isolate probes containing this modification by "catching" the hook. The interaction between hook and catching mechanism can be, for example, covalent bonding or ligand/receptor binding of sufficient affinity. Such hooks may include antigens which can be recovered by antibody, biotin which can be recovered by avidin or streptavidin, specific DNA sequences which can be recovered by complementary nucleic acid, or DNA binding proteins (repressors), and specific reactive chemical functionalities which can be recovered by other appropriate reactive groups.

"Hybridization" and "binding" in the context of probes and denatured melted DNA are used interchangeably. Probes which are hybridized or bound to denatured DNA are base paired or "aggregated" to complementary sequences in the polynucleotide. Whether or not a particular probe remains base paired or aggregated with the polynucleotide depends on the degree of complementarity, the length of the probe, and the stringency of the binding conditions. The higher the stringency, the higher must be the degree of complementarity, and/or the longer the probe.

"Klenow fragment" refers to a 76,000 dalton polypeptide obtained by partial proteolytic digestion of DNA polymerase I. This enzyme possesses the 5'--->3' polymerase and 3'--->5'

exonuclease activities, but not the 5'→3' exonuclease activity of DNA polymerase I.

"Label" refers to a modification to the probe nucleic acid which enables the user to identify the labelled nucleic acid in the presence of unlabelled nucleic acid. Most commonly, this is the replacement of one or more atoms with radioactive isotopes. However, other labels may be substituted for the isotopes as, for example, covalently attached chromophores, fluorescent moieties, enzymes, antigens, groups with specific reactivity, chemiluminescent moieties, and electrochemically detectable moieties.

"Ligase" refers to an enzyme which catalyses the formation of a phosphodiester bond at the site of a single-stranded break in duplex DNA. The ligase enzyme also catalyses the covalent linkage of duplex DNA; blunt end to blunt end, or one cohesive end to another complementary cohesive end.

"Ligase Chain Reaction (LCR)" refers to the amplification of a oligonucleotide ligation product. For example, if oligonucleotides are designed such that the DNA products of one cycle can become the DNA substrates of the next cycle, repeating such cycles will cause an exponential amplification of the DNA (a "chain reaction"). As a thermophilic ligase enzyme is capable of remaining active during many DNA melting and cooling cycles, this allows a DNA amplification to occur rapidly and automatically in a single reaction vessel subject to many thermal cycles in which the oligonucleotide ligation product is amplified.

"Ligase detection reaction (LDR)" refers to the use of two adjacent oligonucleotides for the detection of specific sequences with the aid of a thermophilic ligase with linear product amplification.

- 5 "Ligase DNA sequence" refers to the DNA sequence in *Thermus aquaticus* HB8 for the thermophilic ligase of the present invention which comprises, at the amino terminus of the ligase protein, the following nucleic acid sequence:

10 TCGGAATAGG GGATGCGCCC CTAGTCCAAG GGAAAGTATA GCCCAAGGTG  
CACTAGGGCC

ATG ACC CTG GAA GAG GCG AGG AAG CGG GTA AAC GAG TTA CGG GAC  
CTC ATC CGC TAC CAC AAC TAC CGC TAC TAC GTC CTG GCG GAC CCG  
GAG ATC TCC GAC GGC GAG TAC GAC CGG CTT CTT AGG GAG CTC AAG  
15 GAG CTT GAG GAG CGC TTC CCC GAG CTC AAA AGC CCG GAC TCC CCC  
ACC CTT CAG GTG GGG GCG AGG CCT TTG GAG GCC ACC TTC CGC CCC  
GTC CGC CAC CCC ACC CGC ATG TAC TCC TTG GAC AAC GCC TTT AAC  
CTT GAC GAG CTC AAG GCC TTT GAG GAG CGG ATA GAA CGG GCC CTG  
GGG CGG AAG GGC CCC TTC GCC TAC ACC GTG GAG CAC AAG GTG GAC  
20 GGG CTT TCC GTG AAC CTC TAC TAC GAG GAG GGG GTC CTG GTC TAC  
GGG GGC ACC GGC GGG GAC GGG GAG GTG GGG GAG GAG GTC ACC CAG  
AAC CTC CTC ACC ATC CCC ACC ATC CCG AGG AGG CTC AAG GGG GTG  
CCG GAG CGC CTC GAG GTC CGG GGG GAG GTC TAC ATG CCC ATA GAG  
GCC TTC CTC CGG CTC AAC GAG GAG CTG GAG GAG CGG GGG GAG AGG  
25 ATC TTC AAA AAC CCT AGG AAT GCG GCG GCG GGT TCC TTA AGG CAA  
AAA GAC CCC CGC ATC ACC GCC AAG CGG GGC CTC AGG GCC ACC TTC  
TAC GCC TTA GGG CTT GGG CTG GAG GAG GTG GAG AGG GAA GGG GTG  
GCG ACC CAG TTT GCC CTC CTC CAC TGG CTC AAG GAA AAA GGC TTC  
CCC GTG GAG CAC GGC TAC GCC CGG GGC GTG GGG GCG GAA GGG GTG  
30 GAG GCG GTC TAC CAG GAC TGG CTC AAG AAG CGG CGG GCG CTT CCC  
TTT GAG GCG GAC GGG GTG GTG GTG AAG CTG GAC GAG CTT GCC CTT  
TGG CGG GAG CTC GGC TAC ACC GCC CGC GGC CCC CGG TTC GCC ATC

5   GCC TAC AAG TTC CCC GCC GAG GAG AAG GAG ACC CGG CTT TTG GAC  
      GTG GTC TTC CAG GTG GGG CGC ACC GGG CGG GTG ACC CCC GTG GGG  
      ATC CTC GAG CCC GTC TTC CTA GAG GGC AGC GAG GTC TCC CGG GTC  
      ACC CTG CAC AAC GAG AGC TAC ATA GAG GAG TTG GAC ATC CGC ATC  
 10   GGG GAC TGG GTT TTG GTG CAC AAG GCG GGC GGG GTC ATC CCC GAG  
      GTC CTC CGG GTC CTC AAG GAG AGG CGC ACG GGG GAG GAA AGG CCC  
      ATT CGC TGG CCC GAG ACC TGC CCC GAG TGC GGC CAC CGC CTC CTC  
      AAG GAG GGG AAG GTC CAC CGC TGC CCC AAC CCC TTG TGC CCC GCC  
      AAG CGC TTT GAG GCC ATC CGC CAC TTC GCC TCC CGC AAG GCC ATG  
 15   GAC ATC CAG GGC CTG GGG GAA AAG CTC ATT GAG AGG CTT TTG GAA  
      AAG GGG CTG GTC AAG GAC GTG GCC GAC CTC TAC CGC TTG AGA AAG  
      GAA GAC CTG GTG GGC CTG GAG CGC ATG GGG GAG AAG AGC GCC CAA  
      AAC CTC CTC CGC GAG ATA GAG GAG AGC AAG AAA AGA GGC CTG GAG  
      CGC CTC CTC TAC GCC TTG GGG CTT CCC GGG GTG GGG GAG GTC TTG  
 20   GCC CGG AAC CTG GCG GCC CGC TTC GGG AAC ATG GAC CGC CTC CTC  
      GAG GCC AGC CTG GAG GAG CTC CTG GAG GTG GAG GAG GTG GGG GAG  
      CTC ACG GCG AGG GCC ATC CTG GAG ACC TTG AAG GAC CCC GCC TTC  
      CGC GAC CTG GTA CGG AGG CTC AAG GAG GCG GGG GTG GAG ATG GAG  
      GCC AAG GAG AAG GGC GGG GAG GCC CTT AAA GGG CTC ACC TCC GTG  
      ATC ACC GGG GAG CTT TCC CGC CCC CGG GAA GAG GTG AAG GCC CTC  
      CTA AGG CGC CTC GGG GCC AAG GTG ACG GAC TCC GTG AGC CGG AAG  
      ACG AGC TAC CTC GTG GTG GGG GAG AAC CCG GGG GAG AAC CCG GGG  
      AGC AAG CTG GAG AAG GCC AGG GCC CTC GGG GTC CCC ACC CTC ACG  
 25   GAG GAG GAG CTC TAC CGG CTC CTG GAG GCG CGG ACG GGG AAG AAG  
      GCG GAG GAG CTC GTC TAA AGGCTTCC.

The corresponding amino acids are:

30   Met Thr Leu Glu Glu Ala Arg Lys Arg Val Asn Glu Leu Arg Asp  
      Leu Ile Arg Tyr His Asn Tyr Arg Tyr Tyr Val Leu Ala Asp Pro  
      Glu Ile Ser Asp Ala Glu Tyr Asp Arg Leu Leu Arg Glu Leu Lys  
      Glu Leu Glu Glu Arg Phe Pro Glu Leu Lys Ser Pro Asp Ser Pro  
      Thr Leu Gln Val Gly Ala Arg Pro Leu Glu Ala Thr Phe Arg Pro  
      Val Arg His Pro Thr Arg Met Tyr Ser Leu Asp Asn Ala Phe Asn  
      Leu Asp Glu Leu Lys Ala Phe Glu Glu Arg Ile Glu Arg Ala Leu  
      Gly Arg Lys Gly Pro Phe Ala Tyr Thr Val Glu His Lys Val Asp

Gly Leu Ser Val Asn Leu Tyr Tyr Glu Glu Gly Val Leu Val Tyr  
Gly Ala Thr Arg Gly Glu Gly Glu Val Gly Glu Glu Val Thr Gln  
Asn Leu Leu Thr Ile Pro Thr Ile Pro Arg Arg Leu Lys Gly Val  
Pro Glu Arg Leu Glu Val Arg Gly Glu Val Tyr Met Pro Ile Glu  
5 Ala Phe Leu Arg Leu Asn Glu Glu Leu Glu Glu Arg Gly Glu Arg  
Ile Phe Lys Asn Pro Arg Asn Ala Ala Ala Gly Ser Leu Arg Gln  
Lys Asp Pro Arg Ile Thr Ala Lys Arg Gly Leu Arg Ala Thr Phe  
Tyr Ala Leu Gly Leu Gly Leu Glu Glu Val Glu Arg Glu Gly Val  
Ala Thr Gln Phe Ala Leu Leu His Trp Leu Lys Glu Lys Gly Phe  
10 Pro Val Glu His Gly Tyr Ala Arg Ala Val Gly Ala Glu Gly Val  
Glu Ala Val Tyr Gln Asp Trp Leu Lys Lys Arg Arg Ala Leu Pro  
Phe Glu Ala Asp Gly Val Val Val Lys Leu Asp Glu Leu Ala Leu  
Try Arg Glu Leu Gly Tyr Thr Ala Arg Ala Pro Arg Phe Ala Ile  
Ala Tyr Lys Phe Pro Ala Glu Glu Lys Glu Thr Arg Leu Leu Asp  
15 Val Val Phe Gln Val Gly Arg Thr Gly Arg Val Thr Pro Val Gly  
Ile Leu Glu Pro Val Phe Leu Glu Gly Ser Glu Val Ser Arg Val  
Thr Leu His Asn Glu Ser Tyr Ile Glu Glu Leu Asp Ile Arg Ile  
Gly Asp Trp Val Leu Val His Lys Ala Gly Gly Val Ile Pro Glu  
Val Leu Arg Val Leu Lys Glu Arg Arg Thr Gly Glu Glu Arg Pro  
20 Ile Arg Trp Pro Glu Thr Cys Pro Glu Cys Gly His Arg Leu Leu  
Lys Glu Gly Lys Val His Arg Cys Pro Asn Pro Leu Cys Pro Ala  
Lys Arg Phe Glu Ala Ile Arg His Phe Ala Ser Arg Lys Ala Met  
Asp Ile Gln Gly Leu Gly Glu Lys Leu Ile Glu Arg Leu Leu Glu  
Lys Gly Leu Val Lys Asp Val Ala Asp Leu Tyr Arg Leu Arg Lys  
25 Glu Asp Leu Val Gly Leu Glu Arg Met Gly Glu Lys Ser Ala Gln  
Asn Leu Leu Arg Gln Ile Glu Glu Ser Lys Lys Arg Gly Leu Glu  
Arg Leu Leu Tyr Ala Leu Gly Leu Pro Gly Val Gly Glu Val Leu  
Ala Arg Asn Leu Ala Ala Arg Phe Gly Asn Met Asp Arg Leu Leu  
Glu Ala Ser Leu Glu Glu Leu Leu Glu Val Glu Glu Val Gly Glu  
30 Leu Thr Ala Arg Ala Ile Leu Glu Thr Leu Lys Asp Pro Ala Phe  
Arg Asp Leu Val Arg Arg Leu Lys Glu Ala Gly Val Glu Met Glu  
Ala Lys Glu Lys Gly Gly Glu Ala Leu Lys Gly Leu Thr Phe Val  
Ile Thr Gly Glu Leu Ser Arg Pro Arg Glu Glu Val Lys Ala Leu  
Leu Arg Arg Leu Gly Ala Lys Val Thr Asp Ser Val Ser Arg Lys

Thr Ser Tyr Leu Val Val Gly Glu Asn Pro Gly Ser Lys Leu Glu  
Lys Ala Arg Ala Leu Gly Val Pro Thr Leu Thr Glu Glu Glu Leu  
Tyr Arg Leu Leu Glu Ala Arg Thr Gly Lys Lys Ala Glu Glu Leu  
Val

5 "Ligating" refers to covalently attaching polynucleotide  
sequences together to form a single sequence. This is typically  
performed by treatment with a ligase which catalyzes the  
formation of a phosphodiester bond between the 5' end of one  
sequence and the 3' end of the other. However, in the context of  
10 the invention, the term "ligating" is also intended to encompass  
other methods of covalently attaching such sequences, e.g., by  
chemical means. The terms "covalently attaching" and "ligating"  
may be used interchangeably.

"Nick closing activity" refers to covalent linkage of  
15 adjacent strands of DNA. It may be used to assay for ligase  
activity by virtue of converting open circular DNA (OCDNA) to  
covalently closed circular DNA (CCCDNA) and determining the  
speed at which the specimen DNA migrates on an ethidium  
bromide stained agarose gel (OCDNA migrates slower than  
20 CCCDNA).

"Oligonucleotide" refers to a molecule comprised of two or  
more deoxyribonucleotides or ribonucleotides, preferably more  
than three. Its exact size will depend on the ultimate function or  
use of the oligonucleotide. The oligonucleotide may be derived  
25 synthetically or by cloning.

"Operably linked" refers to juxtaposition such that the  
normal function of the components can be performed. Thus, a

coding sequence "operably linked" to control sequences refers to a configuration wherein the coding sequences can be expressed under the control of the control sequences.

"Overproducer strain" refers to a strain of bacteria or other  
5 host cell that may be induced to overproduce a particular enzyme or chemical substance.

"Polymerase" refers to enzymes which catalyze the assembly of deoxyribonucleotides into DNA.

"Polymerase chain reaction (PCR)" refers to a patented  
10 process (described in United States Patents 4,683,202 and 4,683,195) for the exponential amplification of a specific DNA fragment by utilizing two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in a target DNA. The process consists of a repetitive series of cycles  
15 involving template denaturation, primer annealing, and the extension of the annealed primers by *Taq* DNA polymerase.

"Probe" refers to an oligonucleotide designed to be sufficiently complementary to a sequence in a denatured nucleic acid to be probed (in relation to its length) to be bound under  
20 selected stringency conditions. "Contiguous probe" describes a probe which is complementary to the contiguous portion.

"Diagnostic probe" describes a probe which is complementary to the diagnostic portion. "Target probe" describes a probe which is complementary to the target sequence and is made by covalently  
25 attaching (ligating) the diagnostic probe and the contiguous probe.



"Reporter group" refers to a group that signifies the presence of a particular moiety (see "label").

"Restriction endonucleases" refers to those enzymes which cut DNA by recognizing specific sequences internal to the molecule and subsequently cutting the DNA in both strands at sites either within or outside of the recognition sequence.

"Sticky end ligation" refers to the covalent linkage of two ends of DNA that contain complementary 5' or 3' single strand overhangs which are usually, but not limited to, one to five nucleotides in length.

"Stringency" refers to the combination of conditions to which nucleic acids are subject that cause the double stranded DNA to dissociate into component single strands; among these are pH extremes, high temperature, and salt concentration. "High stringency" refers to the conditions, specifically hybridization and washing, which are sufficient to allow for the detection of unique sequences using an oligonucleotide probe or closely related sequence under standard Southern hybridization protocols [as described in J. Mol. Biol. 98:503 (1975)].

" $T_m$ " refers to the temperature at which two complementary strands of DNA unwind and separate. This is a function of the single stranded DNA length and its base composition - for small fragments, an approximate value of  $T_m$  in  $^{\circ}\text{C}$  is equal to  $4(\text{G}+\text{C}) + 2(\text{A}+\text{T})$ . For example, an oligonucleotide which has 5G, 7C, 5A, and 4T bases has a temperature of  $4(5+7) + 2(5+4)$  or  $66^{\circ}\text{C}$ .

"Target sequence" refers to a nucleic acid sequence, the presence or absence of which is desired to be detected. In the context of a preferred application of the method according to the present invention, it is a sequence which forms part of a coding  
5 region in a gene associated with a genetic disease, such as sickle-cell anemia. In many such diseases, the presence of the genetic aberration is characterized by small changes in the coding sequence; most frequently, normal individuals have sequences which differ by one nucleotide from the corresponding sequences  
10 present in individuals with the genetic "deficiency." In the method according to the present invention, either the normal or altered sequence can be used as the target sequence.

"Thermophilic enzyme" refers to an enzyme which functions at high temperatures of 50 to 90°C; some may survive brief  
15 exposure to temperatures of 94 to 100°C at which normal enzymes denature and thus become inactive.

"Thermostable ligase" refers to an enzyme which is stable to heat, is heat resistant, and catalyzes (facilitates) ligation, at high temperatures of 50 to 90°C, of adjacent oligonucleotides in  
20 the proper manner to form a product which is complementary to the target nucleic acid strand. Generally, the enzyme activates the 5' end of one oligonucleotide and links this to the 3' strand of an adjacent DNA molecules. There may, however, be thermostable enzymes which use other mechanisms to covalently attach  
25 adjacent oligonucleotides. Thermostable ligase can, under the proper conditions, covalently link a number of different nucleic

acid substrates at high temperatures of 50 to 90°C, such as closing "nicks" in DNA, and sticky end and blunt end ligations.

The thermostable enzyme according to the present invention must satisfy a single criterion to be effective for the amplification reaction, i.e., the enzyme must not become irreversibly denatured (inactivated) when subjected to the elevated temperatures for the time necessary to effect denaturation of double-stranded nucleic acids. By "irreversible denaturation" as used in this connection, is meant a process bringing about a permanent and complete loss of enzymatic activity. The heating conditions necessary for denaturation will depend, e.g., on the buffer salt concentration and the length and nucleotide composition of the nucleic acids being denatured, but typically range from about 85°C, for shorter oligonucleotides, to about 105°C for a time depending mainly on the temperature and the nucleic acid length, typically from about 0.25 minutes for shorter oligonucleotides, to 4.0 minutes for longer pieces of DNA. Higher temperatures may be tolerated as the buffer salt concentration and/or GC composition of the nucleic acid is increased. Preferably, the enzyme will not become irreversibly denatured at about 90 to 100°C. The thermostable enzyme according to the present invention has an optimum temperature at which it functions that is greater than about 45°C, probably between 50 and 90°C, and optimally between 60 and 80°C.

A more thorough and complete understanding of the cloning of the thermophilic ligase sequence and the use of this enzyme in

the thermophilic ligase mediated DNA amplification procedure for the detection of single base pair sequence differences in genetic diseases can be obtained by reference to the following figures and examples which are presented by way of illustration only and are not intended, nor should they be considered, to limit the scope of the claimed invention.

With specific reference to the figures,

FIG. 1 is a depiction of plasmids pDZ1 and pDZ7;

FIG. 2 is a flow chart of the Ligase Chain Reaction (LCR) according to the present invention;

FIG. 3 is an autoradiogram demonstrating the specificity of *T. aquaticus* thermophilic ligase under both LDR and LCR amplification conditions according to the present invention;

FIG. 4 is an autoradiogram demonstrating LCR amplification at different target concentrations;

FIG. 5 is an autoradiogram demonstrating the detection of  $\beta$  globin alleles using human genomic DNA.

FIG. 6 is an overview of an ELISA based oligonucleotide ligation assay according to the present invention.

FIG. 7 is a photographic representation of SDS-10% polyacrylamide gel electrophoresis of the thermostable ligase, according to the present invention, at different stages of purification.

FIG. 8 is a second photographic representation of SDS-10%

polyacrylamide gel electrophoresis of the thermostable ligase, according to the present invention, at different stages of purification.

FIG. 9 is a depiction of three clones prepared in accordance with the present invention.

In Fig. 7, lanes A and G represent marker proteins (molecular weights are given in kd); B represents whole cells after induction; C represents crude supernatant after sonication; D represents pooled DEAE flow-through after heat treatment; and E and F represent fractions 23 and 24 after phosphocellulose chromatography. In Fig. 8, lanes A and H represent marker proteins (molecular weights are given in kd); B represents whole cells after induction; C represents crude supernatant after sonication; D represents pooled DEAE flow-through after heat treatment; E represents fraction 23 after phosphocellulose chromatography; F represent fraction 23 incubated with nicked DNA in ligase buffer in the absence of NAD; and G represents fraction 23 incubated with NAD in ligase buffer in the absence of nicked DNA. In Fig. 8, the higher molecular weight ligase (approximately 81 kd) is the adenylated form, while lower molecular weight ligase (approximately 78 kd) is non-adenylated.

The plasmids depicted in Fig. 1 have been deposited with, and accepted by, a collection agency under the Budapest Treaty deposit rules. Plasmid pDZ1 has been incorporated within a host bacteria (*E. coli* strain AK53), deposited with the American Type Culture Collection, and granted the collection number ATCC No.

68307. Plasmid pDZ7 has been incorporated within a host bacteria (*E. coli* strain AK53), deposited with the American Type Culture Collection, and granted the collection number ATCC No. 68308.

5 While other methods may be used, in general, the production of the thermophilic ligase according to the present invention will be by recombinant means which typically involve the following:

First, a DNA is obtained which encodes the mature (as used herein the term includes all muteins) enzyme or a fusion of the  
10 thermophilic ligase to an additional sequence that does not destroy its activity or to an additional sequence cleavable under controlled conditions to give an active protein. If the sequence is uninterrupted by introns, it is suitable for expression in any host. However, the sequence should be in an excisable and recoverable  
15 form. Using PCR technology, for example, most DNA sequences coding for enzymes may be amplified and hence recovered in an "excised" form.

The excised or recovered coding sequence is then placed in operable linkage with suitable control sequences in a replicable  
20 expression vector which is used to transform a suitable host. The transformed host is then cultured under suitable conditions to effect the production of the recombinant thermophilic ligase, and the ligase isolated and purified by known means.

Each of the above procedures may be accomplished in a  
25 variety of ways. For example, the desired coding sequences may be obtained from genomic fragments and used directly in

appropriate hosts; the constructions for expression vectors operable in a variety of hosts are made using appropriate replicons and control sequences; and suitable restriction sites may, if not normally available, be added to the ends of the coding  
5 sequence so as to provide an excisable gene to insert into the appropriate vector.

The control sequences, expression vectors, and transformation methods are dependent on the type of host cell used to express the gene. Generally, bacterial hosts are the most  
10 efficient and convenient for the production of recombinant proteins and therefore preferred for the expression of the thermophilic ligase according to the present invention. However, other hosts such as yeast, plant, and insect or mammalian cells may also be used if convenient. For the purposes of the present  
15 invention, one source of the host cell is considered to be equivalent to any other available and suitable host cell source.

## EXAMPLE I

(growth of *T. aquaticus* strain HB8 and isolation of DNA)

DNA was isolated from *Thermus thermophilus* strain HB8 (ATCC No. 27634). This strain has recently been reclassified as  
5 *Thermus aquaticus* strain HB8 [see Arch. Microbiol 117:189 (1978)].

Cells were grown overnight at 75°C in a water bath shaker in TAB broth [see Nuc. Acids Res., pgs 6795-6804 (1981)] (which contains per liter, 5 g Bacto™-tryptone, 3 g yeast extract, 2 g  
10 NaCl, and 1 g dextrose) adjusted to pH 7.2 - 7.5 with NaOH, and harvested by centrifugation to yield 3.1 g wet weight from 800 ml of media. Cells were resuspended in 15 ml of 50 mM Tris pH 8.0 buffer containing 50 mM EDTA and 15 mg egg white lysozyme. The resuspended cells were lysed by the addition of 2 ml of 10%  
15 (weight/volume) sodium dodecyl sulfate followed by incubation at 37°C for 15 minutes and two repeated cycles of freezing at -50°C and thawing at 37°C. The aqueous solution was extracted sequentially with equal volumes of aqueous phenol (preequilibrated to pH 7.5 with sodium borate), followed by  
20 phenol/chloroform, and finally chloroform.

Nucleic acids were precipitated by mixing with 2 volumes of 95% ethanol, chilling to -50°C for 15 min., and pelleted by centrifugation. After removal of the supernatant and drying the pellet, nucleic acids were resuspended in 1 ml TE buffer (10 mM  
25 Tris HCl, pH 8.0, containing 1 mM EDTA). RNA was digested by the addition of 100 µg RNase A to each ml of suspension, and the



mixture incubated at 37°C for 1 hr. DNA was precipitated by adding 1/10th vol. of 3 M sodium acetate and 3 vol. of 100% ethanol, chilled to -50°C for 15 min., pelleted by centrifugation, washed with 70% ethanol, and finally resuspended in TE buffer at  
5 a final concentration of 2 mg/ml.

Although DNA utilized in the example given above was isolated from *Thermus aquaticus*, the resultant thermophilic ligase having the necessary properties according to the present invention may have as its initial source DNA isolated from other

5 *Thermus* species or other thermophilic bacteria, phages, or viruses.

DNA isolated from *T. aquaticus* strain HB8 cannot be cleaved by the restriction endonucleases *Taq* I (whose recognition sequence is TCGA) or EcoRI (whose recognition sequence is

10 GAATTC). The inability to cleave certain sequences is a consequence of protective methylation [see H. O. Smith and S. V. Kelly, DNA Methylation: Biochemistry and Biological Significance, eds. Razin, Cedar and Riggs, p 39 - 71, Springer-Verlag Inc., New York (1987)] at the N6 position of adenine residues. Previous

15 investigators [see J. Bact. 169:3243 (1987)] have shown that there is a gene, termed *mrr*, which restricts adenine methylated DNA of the form G-6MeANTC and CTGC-6MeAG. In the cloning of the *Taq* I restriction endonuclease and methylase, several *E. coli* strains were found to restrict the TCGA methylated DNA, an

20 affect originally (but incorrectly) attributed to the *mrr* gene [see Gene 56:13 (1987) and Nuc. Acid Res. 15:9781 (1987)]. Recent work conducted at the Cornell University Medical College has shown the presence of an additional gene, besides *mrr* which encodes a protein that restricts TCGA methylated DNA. Briefly,

25 strains containing a Tn5 ( $Km^R$ ) transposon disrupting the *mrr* gene were [see J. Bact. 169:3243 (1987)] used for transduction

[according to J. H. Miller in Experiments in Molecular Genetics, Cold Spring Harbor Laboratory, pp 201-205 (1972)] of the Km<sup>R</sup> marker into several strains of *Escherichia coli* that resulted in strain converts to a *mrr*<sup>-</sup> (defective *mrr* protein) genotype. None  
5 of these transduced strains could tolerate the *Taq* methylase gene, indicating there is a second gene responsible for the restriction of TCGA methylated DNA. Thus, one of the first necessary requirements (which prior to the present invention had not been apparent) for the making of the present invention was  
10 the selection of an *E. coli* strain which would not heavily restrict TCGA methylated DNA.

In the present invention, a derivative of the RRI strain of *E. coli* which could tolerate the *Taq* methylase gene and which contained a Tn10 (Tc<sup>R</sup>) transposon was transduced to a *ligts7*  
15 strain [N3098, see Wilson and Murray, J. Mol. Biol. (1979) and J. Mol. Biol. 77:531 (1973)] to create *E. coli* strain AK76. This strain has been deposited in the American Type Culture Collection, and has been granted the collection number ATCC No. 55032. This strain contains a temperature sensitive ligase gene,  
20 such that at 42°C the strain cannot grow. This strain can tolerate the *Taq* methylase gene, and other methylated DNA, especially the DNA isolated from *T. aquaticus*. Since it also has a temperature sensitive ligase gene, it could be used as a host for the cloning of a functional *T. aquaticus* ligase gene by selecting for growth at  
25 42°C.

Cloning of the *T. aquaticus* ligase gene was based on a positive selection scheme similar to that described by Wilson and Murray. The approach was to construct libraries of *T. aquaticus* DNA inserted into a suitable vector. These libraries were then  
5 introduced via transformation into a *ligts7* *E. coli* strain that did not restrict methylated *T. aquaticus* DNA, such as strain AK76. These cells were then grown at the nonpermissive temperature, that is at 42°C. Any survivors could be (i) revertants to a *lig+* phenotype; (ii) second site revertants that increase expression of  
10 the defective *E. coli* ligase gene product; (iii) a cloned piece of *T. aquaticus* DNA that increases expression of the defective *E. coli* ligase gene product; or (iv) a cloned piece of *T. aquaticus* DNA that contains the *T. aquaticus* ligase gene.

For the desired last alternative to work, it is necessary  
15 that (i) the entire ligase gene is cloned; (ii) that either the endogenous control sequences for *T. aquaticus* ligase expression function in *E. coli*, or that exogenous vector control sequences are sufficiently close to the amino terminus and the ligase gene is cloned in the correct orientation to allow for proper expression in  
20 *E. coli*; (iii) the *T. aquaticus* ribosome binding site works in *E. coli*; and (iv) the *T. aquaticus* ligase is active enough at 42°C, and the amount synthesized is sufficient to complement ligase function in *E. coli* without interfering with other processes.

Construction of the suitable libraries used in the present  
25 invention utilized conventional vectors containing desired control sequences, and standard restriction endonuclease and ligation

techniques. Purified plasmid DNA, *T. aquaticus* DNA sequences, or synthesized oligonucleotides for use in the present invention, were cleaved, tailored, and religated in the form desired also by conventional techniques.

5       The selection of a suitable vector for use in the present invention is more than a mere matter of selecting a vector among the many which exist and have been used in the past. High copy number derivatives of pUC plasmids [see for example, C. Yanisch-Peron et al., Gene 33:103 (1985), or J. Vieira et al., Gene 19:259  
10 (1982)] are actually somewhat unstable when grown at 42°C. Low copy plasmids such as pBR322 derivatives pFBI 1, 2, 13, 14 and 15 [see F. Barany, Proc. Natl. Acad. Sci. USA 82:4202 (1985)] may not produce enough enzyme to complement the ligase defect. In making the present invention, 18 different libraries using 3  
15 different sets of vectors were constructed. The successful clone was derived from the vector pTZ18R [see D. A. Mead et al., Protein Engineering 1:67 (1986)], although other vectors may also be utilizable.

Generally, site-specific DNA cleavage, as more particularly  
20 described in the following example, is performed by treating the DNA with a suitable restriction enzyme under conditions which are generally understood in the art, and the particulars of which are specified by the manufacturers of these commercially available restrictions enzymes. In general, about 1 µg of plasmid  
25 or DNA sequence is cleaved by two to ten units of enzyme in about 20 µl of buffer solution. Incubation times of about one to two

hours at about 37°C are preferable, although variations in both the time and temperature can be tolerated. After each incubation, protein is removed by extraction with phenol/chloroform, and may be followed by a further extraction. The nucleic acids are  
5 recovered by precipitation with ethanol. If desired, size separations of the cleaved fragments may be performed by polyacrylamide or agarose gel electrophoresis using standard techniques.

## EXAMPLE II

### (site specific cleavage)

Site-specific cleavage of both plasmid and *T. aquaticus* DNA was performed using commercially available restriction  
5 endonucleases in standard buffers.

In general, about 10 µg of plasmid or *T. aquaticus* DNA was cleaved in 100 µl of buffer solution by the addition of 20 to 100 units of the appropriate restriction endonuclease, and incubating the mixture at 37°C for 1 to 2 hrs.

10 After each incubation, protein was removed by sequential extractions with phenol (2x), n-butanol (2x), and the nucleic acid was recovered by precipitation with ethanol.

Construction of suitable vectors containing the desired coding and control sequences employs conventional ligation and restriction techniques. Briefly, isolated plasmids, DNA sequences, or synthesized oligonucleotides are cleaved, tailored, and religated in the form desired.

The restriction endonucleases utilized for cleavage of the specific libraries used in accordance with the procedure outlined in Example II were BamHI, SacI, KpnI (Asp718), PstI, HindIII, and SmaI, however, other endonucleases or partial digests with SauIII A, for example, could have been used. Due to adenosine methylation, the commonly utilized restriction endonucleases EcoRI, SalI or XhoI were not used since DNA from *T. aquaticus* strain HB8 could not be cleaved by these enzymes.

Restriction fragments resulting from the procedure outlined in Example II containing 5' overhangs may be blunt ended by filling in with DNA polymerase I large (Klenow fragment) in the presence of the four deoxynucleotide triphosphates using incubation times of about 15 to 30 minutes at 37°C in 50 mM Tris pH 7.6 buffer containing 50 mM NaCl, 10 mM MgCl<sub>2</sub>, 10 mM DTT, and 50 - 100 μM deoxynucleotide triphosphates. The Klenow fragment will fill in at 5' sticky ends. If 3' overhangs are generated, they may be chewed back with mung bean nuclease. After treatment with Klenow, the mixture is extracted with phenol/chloroform and precipitated with ethanol. Subsequent treatment under appropriate conditions with S1 nuclease results in hydrolysis of any single stranded portion. These conventional procedures may



be used for cloning any fragment into a (blunt end) site within the vector.

## EXAMPLE III

## (vector construction)

In vector constructions, the linearized vector is commonly treated with a phosphatase enzyme (or alternatively with a second nearby restriction endonuclease) to prevent recircularization of the vector in the absence of insert DNA. For example, a sample of BamHI (5' overhang) or SacI (3' overhang) DNA (9 µg) in 150 µl 50 mM Tris HCl buffer at pH 8.0 and containing 10 mM MgCl<sub>2</sub> and 6 mM mercaptoethanol in the presence of Na<sup>+</sup> may be treated with Calf Intestine Alkaline Phosphatase (CIAP, 22 units) at 37°C for 15 min., followed by incubation at 50°C for 30 min. to remove phosphate groups from either 5' or 3' overhangs. Alternatively, Bacterial Alkaline Phosphatase (BAP, 10 units) may be used in 150 µl 10 ml Tris HCl in the presence of Na<sup>+</sup> and Mg<sup>++</sup> and incubating at 60°C for about 1 hr. CIAP may be subsequently denatured by the addition of EDTA and EGTA to chelate divalent cations, and heating to 65°C for 15 min. Either CIAP or BAP protein is then removed by sequential extractions with phenol (2x), n-butanol (2x), and nucleic acid recovered by precipitation with ethanol.

The effectiveness of the phosphatase step is assayed by comparison of the number of transformants generated when vector is religated in the absence or presence of insert DNA. Typical results of from 10 to 100 fold more transformations when insert DNA is present is indicative that the vector DNA has been properly phosphatased.

## EXAMPLE IV

## (ligations)

Ligations were performed in 30-100  $\mu$ l volumes using 1-2  $\mu$ g linearized and phosphatased vector made as previously described. 2-4  $\mu$ g *T. aquaticus* DNA cut with a restriction endonuclease generating the same ends as the vector, in 50 mM Tris HCl buffer at pH 8.0 and containing 10 mM  $MgCl_2$ , 1 mM EDTA, 1 mM ATP, 6 mM mercaptoethanol and from 3 to 7 (Weiss) units of T4 ligase, by incubating at either 4 or 15°C overnight. After ligation, EDTA was added, the T4 ligase inactivated by heating the solution to 65°C for 15 min., and nucleic acids recovered by ethanol precipitation.

Ligation mixtures were introduced into a suitable host such as *E. coli* strains RR1, AK53 or AK76 - the last one suitable for immediate positive selection of the lig<sup>+</sup> phenotype - via conventional transformation procedures [see Hanahan, J. Mol. Biol. 166:3243 (1987)]. Transformants were selected by plating on ampicillin (or other drugs such as tetracycline or kanamycin depending upon the plasmid used) containing plates. For positive selection of the lig<sup>+</sup> phenotype, AK76 transformants were plated onto SOB plates (made by autoclaving 20 g Bacto™-tryptone, 5 g Bacto™-yeast extract, 0.5 g NaCl, 16 g Bacto™-agar in 1 liter of distilled water adjusted to pH 7.5 with NaOH prior to autoclaving, then adding 20 ml 1 M  $MgSO_4$ ) containing 0.2% maltose, 0.2 mg/ml IPTG (to induce the lac promoter), and 50  $\mu$ g/ml ampicillin (to

select the plasmid-containing cells), and grown overnight at 42°C to 42.5°C.

Libraries ranged in size from about 5,000 to 27,000 clones. Given the general estimate that the bacterial chromosome contains about 2,000 to 4,000 kilobases, and the average insert consisted of 5 to 10 kb, it was apparent that several libraries  
5 contained redundant clones.

Mixed plasmid preparations were made from six libraries using conventional techniques [see Methods Enzymol. 100:243 (1983)], and introduced into fresh AK76 cells. Transformants from each library were plated on 6 SOB plates (each plate  
10 receiving between 30,000 and 70,000 clones) and incubated at 42°C. One library produced from 11 to 19 exceedingly small colonies per plate; the remaining libraries produced an occasional large colony.

Individual clones were picked, plasmid DNA prepared using  
15 conventional techniques [see Anal. Biochem. 114:193 (1981)], and analyzed by restriction digestion. All 12 small clones produced a 6.8 kb plasmid containing two BamHI fragments (1.8 and 2.1 kb respectively) cloned within the BamHI site of pTZ18R. One such plasmid has been designated pDZ1 as depicted in Figure 1. By  
20 calculating back to the original library, (of 5,200 clones), it appears that all pDZ1 plasmids derived from a single clone. The large colonies contained plasmids close to the size of the original vector. Therefore, these large colonies are probably revertants of the chromosomal ligts7 gene which contained any plasmid solely  
25 to confer resistance to ampicillin.

Retransforming plasmid pDZ1 into AK76 cells, and selecting at 42°C on SOB plates containing maltose, IPTG, and ampicillin as described in Example IV, again yielded small colonies. Plating fresh transformants on tryptone yeast agar containing ampicillin  
5 did not produce colonies. This result suggests that induction of the lac promoter during plasmid establishment is necessary for production of sufficient quantities of *T. aquaticus* ligase to complement the genetic defect. Once the plasmid has become established in AK76 cells, such clones will give exceedingly  
10 small colonies when streaked and allowed to grow on tryptone yeast plates containing ampicillin at 42°C.

Digestion of pDZ1 with BamHI, followed by religation would scramble the fragments. Transformation of such a ligation mix into AK76, followed by plating at 37°C, i.e. under non-selective  
15 conditions, compared to plating at 42°C, i.e. under selective conditions, yielded 1,000 fold more colonies under non-selective conditions. The starting pDZ1 plasmid yielded only 2 fold more colonies under non-selective than selective conditions. This finding strongly suggests that the presence of both fragments,  
20 and the orientation they are cloned, is necessary for proper expression of *T. aquaticus* ligase.

Although pDZ1 contains several SacI and SmaI sites, it only contains a single (vector derived) PstI, KpnI, or HindIII site. Thus, it would have been expected that a number of ligase clones would  
25 have been isolated from the PstI, KpnI, or HindIII digest libraries. However, the only ligase clone was derived from the partial

BamHI digest library. Although it is not clear why this happened, one conceivable explanation is that other clones did not bring the lac promoter controlling element sufficiently close to the start of the ligase gene to adequately express the ligase protein during  
5 plasmid establishment.

The cloning of *T. aquaticus* ligase as described above will now enable those skilled in the art to clone any thermophilic or thermostable ligase, whether of procaryotic, archebacterial, eukaryotic or phage origin by additional approaches. Accordingly  
10 the cloning of such ligases are within the scope of the present invention.

Such additional approaches to cloning may include, for example, (i) cloning *T. aquaticus* DNA into a red<sup>-</sup> lambda vector and screening for the ability of recombinant phage lambda to form  
15 plaques at 39°C on a ligts7 strain such as AK76 [essentially as generally described in J. Mol. Biol. 132:471 (1979)]; (ii) use of the lambda gt11 phage to express portions of the ligase gene, and subsequently screening with antibodies raised to purified *T. aquaticus* ligase - the positive lambda gt11 clone may then be  
20 used to identify the full length gene by hybridization to other plasmid or phage libraries, essentially as described in the cloning of *T. aquaticus* polymerase [see J. Biol. Chem 264:6427 (1989)];  
90(iii) based upon the ligase DNA sequence, probes can be made that would hybridize to and therefore help to identify and retrieve  
25 other thermostable ligase encoding sequences in a variety of species. Accordingly, portions of the DNA encoding at least five

amino acids from *T. aquaticus* ligase can be replicated, or amplified using PCR techniques, and the denatured or single stranded forms may be used as probes to retrieve additional DNAs encoding a thermophilic or thermostable ligase. Alternatively,  
5 oligodeoxyribonucleotide probes can be synthesized which encode at least five amino acids, and these may be used to retrieve additional DNAs encoding a thermophilic or thermostable ligase.

The selection of a portion of DNA encoding for at least five amino acids is based upon the portion containing fifteen nucleic  
10 acid bases which is more than the statistical minimum length that an oligonucleotide should have in order to find a single complementary sequence in a genome. However, portions slightly smaller (the minimum number in *E. coli* is, for example 12, indicating a portion as small as that encoding for four amino  
15 acids may be acceptable) or larger (the minimum number for higher animals is as high as 19, indicating that a portion encoding for at least seven amino acids may be necessary) [see Oligonucleotides: Antisense Inhibitors of Gene Expression, vol. 12, pages 137 - 140, Macmillan Press Ltd., London (1989)] may be  
20 used to obtain similar results. However, because there may not be a precise match between the nucleotide sequence in the corresponding portions between species, oligomers containing approximately 15 nucleotides are a preferred minimum in order to achieve hybridization under conditions of sufficient stringency to  
25 eliminate false positives; the sequence encoding 5 amino acids



would supply information sufficient for the generation of such probes.

- By way of example, a comparison of the *T. aquaticus* ligase and *E. coli* amino acid sequences reveals an identity between
- 5 amino acids 34-40 (Asp-Ala-Glu-Tyr-Asp-Arg-Leu) at statistically acceptable levels. Using the preferred six amino acid sequence, a degenerate probe of the form GA(C/T)-GC(G/A/T/C)-GA(G/A)-TA(C/T)-GA(C/T)-(C/A)G(G/A/T/C)-(C/T)T could be used to identify and retrieve either of the above ligases.
- 10 The areas of sequence identities between the *Thermophilus* ligase according to the present invention and *E. coli* ligase include the amino acids at the following positions:

	<u>Amino Acid Positions</u>	<u>Consecutive identical aa's</u>
	34 to 40	7
15	57 to 61	5
	137 to 142	6
	168 to 175	8
	199 to 210	12
	212 to 219	8
20	308 to 312	5
	333 to 339	7
	485 to 490	6
	492 to 496	5
	513 to 517	5
25	620 to 624	5

Overall, of the 676 amino acids contained in the ligase according to the present invention, the percent similarity

between the *Thermophilus* ligase and *E. coli* ligase is 66%; the percent identity is 47%.

The construction of an overproducer strain from a cloned and properly oriented gene may be achieved by using procedures which are conventional in the art. The general principle of such construction is to bring an enabling sequence into close proximity to the starting codon of the gene to affect efficient transcription and translation of that gene. There are many promoter systems (including a ribosome binding site [see Proc. Natl. Acad. Sci. USA 78:5543 (1981)]) that have been successfully used to turn on genes, including the lac promoter, the trp promoter [see Gene 20:231 (1982)], the lambda phage P<sub>L</sub> promoter [see Nature 292:128 (1981)], the tac fusion promoter [see Proc. Natl. Acad. Sci. USA 80:21 (1983)], and the T7 phage promoters [see Proc. Natl. Acad. Sci. USA 82:1074 (1985)].

Plasmid pDZ1 contains the *T. aquaticus* ligase gene downstream from both lac and T7 promoters present in the starting vector. There are several methods for removing excess DNA sequences from between the promoters and the gene, including use of Bal31 [see Nucl. Acids Res. 5:1445 (1978)] and ExoIII and Mung Bean or S<sub>1</sub> Nuclease [see Meth. Enzymol. 155:156 (1987)]. However, a somewhat simpler method as described in Example V was used to bring the amino terminus of the *T. aquaticus* ligase gene closer to the two promoters in the present instance.

## EXAMPLE V

(removal of excess DNA from between promoter and gene)

Plasmid pDZ1 was randomly linearized with the restriction endonuclease HinPI (G CGC) and blunt ended with Klenow or  
5 alternatively with CviJI (PuG CPy) [see DNA and Protein Engineering Techniques 1:29 (1988)].

DNA was purified by sequential extractions with phenol (2x), n-butanol (2x), and the nucleic acid recovered by precipitation with ethanol. These randomly linearized plasmids  
10 were then treated with Asp718 which cleaves the polylinker site directly downstream of the two promoters, and blunt ended with Klenow. The resulting fragments were separated via electrophoresis in low melting agarose, sequential slices (including full length linear and progressively smaller DNA  
15 fragments) excised, and the DNA recovered. The DNA fragments were subsequently recircularized by blunt end ligation. This involved overnight incubation at 4°C in 100 µl in 50 mM Tris HCl pH 8.0 buffer containing 10 mM MgCl<sub>2</sub>, 1 mM EDTA, 1 mM ATP, 6 mM mercaptoethanol, and from 3 to 7 Weiss units of T4 ligase.  
20 After ligations, EDTA was added, the T4 ligase inactivated by heat (for 15 min at 65°C), and nucleic acids recovered by ethanol precipitation.

The ligation mixes prepared were introduced into AK76 cells using conventional techniques, and the lig<sup>+</sup> phenotype was  
25 selected at 42°C on SOB plates containing maltose, IPTG, and ampicillin as described previously.

Based upon previous work, plasmids containing deletions between the promoters and the start of the *T. aquaticus* ligase gene would be expected to confer viability under these conditions. Deletions of the vector (promoter regions), or of an essential portion of the ligase gene should not confer viability. Therefore, individual clones were picked, plasmid DNA prepared using conventional methods [see Anal. Biochem. 114:193 (1981)], and analyzed by restriction digestion. Results from this testing found that plasmid pDZ2, pDZ3, pDZ6 and pDZ7 lacked the 1.8 kb BamHI fragment, and contained instead a 1.3, 1.4, 1.2, or 1.2 kb fragment, respectively. All these plasmids re-created the Asp718 site as would be expected with proper blunt end fill-ins and ligations. Single stranded DNA was prepared from these plasmids using conventional techniques [see Nucl. Acids Research 13:1103 (1985), and Protein Engineering 1:64 (1986)], and these were sequenced using the universal "reverse primer" oligonucleotide 5'd(AGCGGATAACAATTTTCACACAGGA)3' and T7 DNA polymerase [see Proc. Natl. Acad. Sci. USA 84:4767 (1987)].

Analysis of the DNA sequence reveals two ATG start codons, the first open reading frame being three codons in length and the second, the ligase DNA sequence, giving a long reading frame. In conjunction with Figure 1, this sequence (including the partial ligase DNA sequence) derived from plasmids pDZ6 and pDZ7 is:

pTZ18R

GGCTCGTATG TTGTGTGGAA TTGTGAGCGG ATAACAATTT

LacZ' T7 Promoter

CACACAGGAA ACAGCTATGA CCATGATTAC GAATTTAATA

5

CGACTCACTA TAGGGAATTC GAGCTCGGTA CCOCAAGGTAEcoRI SacI KpnI

CACTAGGGCC

thermophilic ligase

10 ATG ACC CTG GAA GAG GCG AGG AAG CGG GTA AAC GAG TTA CGG GAC  
 CTC ATC CGC TAC CAC AAC TAC CGC TAC TAC GTC CTG GCG GAC CCG  
 GAG ATC TCC GAC GCC GAG TAC GAC CGG CTT CTT AGG GAG CTC AAG  
 GAG CTT GAG GAG CGC TTC CCC GAG CTC AAA AGC CCG GAC TCC CCC  
 ACC CTT CAG GTG GGG GCG AGG CCT TTG GAG GCC ACC TTC CGC CCC

15 GTC CGC CAC CCC ACC CGC ATG TAC TCC TTG GAC AAC GCC TTT AAC  
 CTT GAC GAG CTC AAG GCC TTT GAG GAG CGG ATA GAA CGG GCC CTG  
 GGG CGG AAG GGC CCC TTC GCC TAC ACC GTG GAG CAC AAG GTG GAC  
 GGG CTT TCC GTG AAC CTC TAC TAC GAG GAG GGG GTC CTG GTC TAC  
 GGG GCC ACC GGC GGG GAC GGG GAG GTG GGG GAG GAG GTC ACC CAG

20 AAC CTC CTC ACC ATC CCC ACC ATC CCG AGG AGG CTC AAG GGG GTG  
 CCG GAG CGC CTC GAG GTC CGG GGG GAG GTC TAC ATG CCC ATA GAG  
 GCC TTC CTC CGG CTC AAC GAG GAG CTG GAG GAG CGG GGG GAG AGG  
 ATC TTC AAA AAC CCT AGG AAT GCG GCG GCG GGT TCC TTA AGG CAA  
 AAA GAC CCC CGC ATC ACC GGC AAG CGG GGC CTC AGG GCC ACC TTC

25 TAC GGC TTA GGG CTT GGG CTG GAG GAG GTG GAG AGG GAA GGG GTG  
 GCG ACC CAG TTT GCC CTC CTC CAC TGG CTC AAG GAA AAA GGC TTC  
 CCC GTG GAG CAC GGC TAC GCC CGG GCC GTG GGG GCG GAA GGG GTG  
 GAG GCG GTC TAC CAG GAC TGG CTC AAG AAG CGG CGG GCG CTT CCC  
 TTT GAG GCG GAC GGG GTG GTG GTG AAG CTG GAC GAG CTT GGC CTT

30 TGG CGG GAG CTC GGC TAC ACC GCC CGC GCC CCC CGG TTC GCC ATC  
 GCC TAC AAG TTC CCC GCC GAG GAG AAG GAG ACC CGG CTT TTG GAC  
 GTG GTC TTC CAG GTG GGG CGC ACC GGG CGG GTG ACC CCC GTG GGG  
 ATC CTC GAG CCC GTC TTC CTA GAG GGC AGC GAG GTC TCC CGG GTC  
 ACC CTG CAC AAC GAG AGC TAC ATA GAG GAG TTG GAC ATC CGC ATC

35 GGG GAC TGG GTT TTG GTG CAC AAG GCG GGC GGG GTC ATC CCC GAG

GTC CTC CGG GTC CTC AAG GAG AGG CGC ACG GGG GAG GAA AGG CCC  
 ATT CGC TGG CCC GAG ACC TGC CCC GAG TGC GGC CAC CGC CTC CTC  
 AAG GAG GGG AAG GTC CAC CGC TGC CCC AAC CCC TTG TGC CCC GCC  
 AAG CGC TTT GAG GCC ATC CGC CAC TTC GCC TCC CGC AAG GCC ATG  
 5 GAC ATC CAG GGC CTG GGG GAA AAG CTC ATT GAG AGG CTT TTG GAA  
 AAG GGG CTG GTC AAG GAC GTG GCC GAC CTC TAC CGC TTG AGA AAG  
 GAA GAC CTG GTG GGC CTG GAG CGC ATG GGG GAG AAG AGC GCC CAA  
 AAC CTC CTC CGC GAG ATA GAG GAG AGC AAG AAA AGA GGC CTG GAG  
 CGC CTC CTC TAC GCC TTG GGG CTT CCC GGG GTG GGG GAG GTC TTG  
 10 GGC CGG AAC CTG GCG GCC CGC TTC GGG AAC ATG GAC CGC CTC CTC  
 GAG GCC AGC CTG GAG GAG CTC CTG GAG GTG GAG GAG GTG GGG GAG  
 CTC ACG GCG AGG GGC ATC CTG GAG ACC TTG AAG GAC CCC GCC TTC  
 CGC GAC CTG GTA CGG AGG CTC AAG GAG GCG GGG GTG GAG ATG GAG  
 GGC AAG GAG AAG GGC GGG GAG GCC CTT AAA GGG CTC ACC TCC GTG  
 15 ATC ACC GGG GAG CTT TCC CGC CCC CGG GAA GAG GTG AAG GCC CTC  
 CTA AGG CGC CTC GGG GCC AAG GTG ACG GAC TCC GTG AGC CGG AAG  
 ACG AGC TAC CTC GTG GTG GGG GAG AAC CCG GGG GAG AAC CCG GGG  
 AGC AAG CTG GAG AAG GGC AGG GCC CTC GGG GTC CCC ACC CTC ACG  
 GAG GAG GAG CTC TAC CGG CTC CTG GAG GCG CGG ACG GGG AAG AAG  
 20 GCG GAG GAG CTC GTC TAA AGGCTTCC

The nucleic acid sequence for the thermophilic ligase  
 according to the present invention corresponds to the amino acid  
 sequence:

Met Thr Leu Glu Glu Ala Arg Lys Arg Val Asn Glu Leu Arg Asp  
 25 Leu Ile Arg Tyr His Asn Tyr Arg Tyr Tyr Val Leu Ala Asp Pro  
 Glu Ile Ser Asp Ala Glu Tyr Asp Arg Leu Leu Arg Glu Leu Lys  
 Glu Leu Glu Glu Arg Phe Pro Glu Leu Lys Ser Pro Asp Ser Pro  
 Thr Leu Gln Val Gly Ala Arg Pro Leu Glu Ala Thr Phe Arg Pro  
 Val Arg His Pro Thr Arg Met Tyr Ser Leu Asp Asn Ala Phe Asn  
 30 Leu Asp Glu Leu Lys Ala Phe Glu Glu Arg Ile Glu Arg Ala Leu  
 Gly Arg Lys Gly Pro Phe Ala Tyr Thr Val Glu His Lys Val Asp  
 Gly Leu Ser Val Asn Leu Tyr Tyr Glu Glu Gly Val Leu Val Tyr  
 Gly Ala Thr Arg Gly Glu Gly Glu Val Gly Glu Glu Val Thr Gln

Asn Leu Leu Thr Ile Pro Thr Ile Pro Arg Arg Leu Lys Gly Val  
 Pro Glu Arg Leu Glu Val Arg Gly Glu Val Tyr Met Pro Ile Glu  
 Ala Phe Leu Arg Leu Asn Glu Glu Leu Glu Glu Arg Gly Glu Arg  
 Ile Phe Lys Asn Pro Arg Asn Ala Ala Ala Gly Ser Leu Arg Gln  
 5 Lys Asp Pro Arg Ile Thr Ala Lys Arg Gly Leu Arg Ala Thr Phe  
 Tyr Ala Leu Gly Leu Gly Leu Glu Glu Val Glu Arg Glu Gly Val  
 Ala Thr Gln Phe Ala Leu Leu His Trp Leu Lys Glu Lys Gly Phe  
 Pro Val Glu His Gly Tyr Ala Arg Ala Val Gly Ala Glu Gly Val  
 Glu Ala Val Tyr Gln Asp Trp Leu Lys Lys Arg Arg Ala Leu Pro  
 10 Phe Glu Ala Asp Gly Val Val Val Lys Leu Asp Glu Leu Ala Leu  
 Try Arg Glu Leu Gly Tyr Thr Ala Arg Ala Pro Arg Phe Ala Ile  
 Ala Tyr Lys Phe Pro Ala Glu Glu Lys Glu Thr Arg Leu Leu Asp  
 Val Val Phe Gln Val Gly Arg Thr Gly Arg Val Thr Pro Val Gly  
 Ile Leu Glu Pro Val Phe Leu Glu Gly Ser Glu Val Ser Arg Val  
 15 Thr Leu His Asn Glu Ser Tyr Ile Glu Glu Leu Asp Ile Arg Ile  
 Gly Asp Trp Val Leu Val His Lys Ala Gly Gly Val Ile Pro Glu  
 Val Leu Arg Val Leu Lys Glu Arg Arg Thr Gly Glu Glu Arg Pro  
 Ile Arg Trp Pro Glu Thr Cys Pro Glu Cys Gly His Arg Leu Leu  
 Lys Glu Gly Lys Val His Arg Cys Pro Asn Pro Leu Cys Pro Ala  
 20 Lys Arg Phe Glu Ala Ile Arg His Phe Ala Ser Arg Lys Ala Met  
 Asp Ile Gln Gly Leu Gly Glu Lys Leu Ile Glu Arg Leu Leu Glu  
 Lys Gly Leu Val Lys Asp Val Ala Asp Leu Tyr Arg Leu Arg Lys  
 Glu Asp Leu Val Gly Leu Glu Arg Met Gly Glu Lys Ser Ala Gln  
 Asn Leu Leu Arg Gln Ile Glu Glu Ser Lys Lys Arg Gly Leu Glu  
 25 Arg Leu Leu Tyr Ala Leu Gly Leu Pro Gly Val Gly Glu Val Leu  
 Ala Arg Asn Leu Ala Ala Arg Phe Gly Asn Met Asp Arg Leu Leu  
 Glu Ala Ser Leu Glu Glu Leu Leu Glu Val Glu Glu Val Gly Glu  
 Leu Thr Ala Arg Ala Ile Leu Glu Thr Leu Lys Asp Pro Ala Phe  
 Arg Asp Leu Val Arg Arg Leu Lys Glu Ala Gly Val Glu Met Glu  
 30 Ala Lys Glu Lys Gly Gly Glu Ala Leu Lys Gly Leu Thr Phe Val  
 Ile Thr Gly Glu Leu Ser Arg Pro Arg Glu Glu Val Lys Ala Leu  
 Leu Arg Arg Leu Gly Ala Lys Val Thr Asp Ser Val Ser Arg Lys  
 Thr Ser Tyr Leu Val Val Gly Glu Asn Pro Gly Ser Lys Leu Glu  
 Lys Ala Arg Ala Leu Gly Val Pro Thr Leu Thr Glu Glu Glu Leu

Tyr Arg Leu Leu Glu Ala Arg Thr Gly Lys Lys Ala Glu Glu Leu  
Val

Translation of the first 60 amino acids of this open reading  
frame (the thermophilic ligase) shows better than 50% homology  
5 to *E. coli* ligase [see Mol. Gen. Genet. 204:1 (1986)] suggesting  
that this long open reading frame represents the start of the *T.*  
*aquaticus* gene. From the genetic results with the BamHI  
fragments, one can conclude that the size of this ligase is  
between 400 and 1,100 amino acids in length. The purified  
10 protein has been reported to have a molecular weight of about  
79,000 [see J. Biol. Chem. 259:10041 (1984)] which is within the  
limits of the genetic results found for the present invention.  
Given that clone pDZ7 produces functional *T. aquaticus* ligase  
(that is it encodes the gene in its entirety), and given the DNA  
15 sequence of the amino terminus, the entire DNA sequence of the  
gene was determined using either manual or automated methods  
as described in the literature [see, for example, Proc. Natl. Acad.  
Sci. 84:4767 (1987); Proc. Natl. Acad. Sci. 86:4076 (1989);  
Science 239:487 (1987); Nature 321:674 (1986); Biotechniques  
20 8:184 (1990); Proc. Natl. Acad. Sci. USA 85:5610 (1988); and Proc.  
Natl. Acad. Sci. USA 85:9436 (1988)].

Plasmids pDZ2, pDZ3, pDZ6 or pDZ7 may be used to construct  
further overproduction vectors using methods common to those  
skilled in biotechnology studies. This may include using  
25 promoters and ribosome binding sites as described above. For  
example, plasmid pDZ7 (see figure 1) may be linearized at its



unique Asp718 site, and excess nucleotides in front of the *T. aquaticus* ligase gene trimmed close to the ATG start codon by the use of Bal31 or a combination of ExoIII and Mung Bean or S<sub>1</sub> Nuclease as described above. This may then be blunt end ligated to a natural enabling sequence (a promoter and translation start sequence) generated in a similar manner, or by a synthetic enabling sequence manufactured for this purpose. In addition, sequences external or internal to the *T. aquaticus* gene may be modified to remove potential RNA structures that may inhibit transcription or translation. These methods have been reported previously to affect overproduction of the thermophilic restriction endonuclease *Taq* I to greater than 30% of soluble *E. coli* proteins [see Gene 65:166 (1988)]. Alternatively, synthetic oligonucleotides may be synthesized such that the start of the *T. aquaticus* ligase gene is fused directly to an enabling sequence using PCR methods [see, for example, Biotechniques 8:178 (1990); Gene 77:51 (1989); and Nucl. Acids Res. 17:723 (1989)].

From the preceeding sequences, it can be seen that there is a *Bgl* II site corresponding to the nucleotides that code for amino acid residues 31-33. With this information, a strong promoter with an optimal Shine-Dalgarno sequence could be inserted in front of this gene using PCR. Two minor caveats need to be considered: (1) attempts to PCR copy the entire gene (3 kb, high GC content) were not always successful, and (2) plasmid pDZ7 had two *Bam* HI and *Bgl* II sites, one each within the ligase gene.

Plasmid pDZ7 was partially digested with both *Bam* HI and *Bgl* II, the correct size smaller linear fragment separated from full length linear by electrophoresis, excised, and purified as described previously. Since *Bam* HI and *Bgl* II produce the same overhang (5' GATC), the linear fragment could be recircularized with T4 ligase, and introduced into *E. coli* strain AK53 via transformation. Several clones had deleted the 0.5 kb *Bam* HI/*Bgl* II fragment resulting in a 5.7 kb plasmid, and one such clone was designated pDZ12. Synthetic oligonucleotides #66, #78, #85, and #94 were synthesized, to allow for fusion of *pho* A promoter [from plasmid pFBT64; see *Gene* 56:13 (1987)] and ribosome binding sequence to the start of the ligase gene using PCR [see *Biotechniques* 8:178 (1990); *Gene* 77:51 (1989); *Gene* 77:61 (1989); and *Nucl. Acids Res.* 17:723 (1989)]. These clones are depicted in Fig. 9, and are:

#66 19 mer; *Pvu* II site to T7 promoter through *pho*A promoter, top strand of plasmid pFBT64 (direction of *Taq*I endonuclease gene):

5' CTG GCT TAT CGA AAT TAA T 3'

#78 32 mer; 5' end complementary to start of *Thermus* ligase gene; 3' end complementary to Shine-Dalgarno side of *pho* A promoter, bottom strand of plasmid pFBT64:

5' CCA GGG TCA TTT TAT TTT CTC CAT GTA CAA AT 3'

#85 33 mer; 5' end complementary to Shine-Dalgarno side of *pho* A promoter; 3' end complementary to start of *T*

*hermus* ligase gene, top strand of plasmid pDZ7 (direction of ligase gene):

5' CAT GGA GAA AAT AAA ATG ACC CTG GAA GAG GCG 3'

#94 18 mer; bottom strand of plasmid pDZ7

5 corresponding to non-translated strand of amino acid residues 40 to 35 of ligase gene, downstream of Bgl II site at amino acid residues 33 to 31:

5' AAG CCG GTC GTA CTC GGC 3'

Briefly, this was accomplished in a single reaction tube in which 400 ng of primers #66 and #78 were added to 200 ng of *Pst* I/*Pvu* II digested pFBT64 containing 50  $\mu$ moles of dATP, cCTP, cGTP, and dTTP each, and 2.5 units *Amplitaq* in 100  $\mu$ l PCR buffer and cycled at 94°C for 1 min, 55°C for 2 min, 72°C for 3 min with 3 sec. extension per cycle for 25 cycles as per the manufacturer's (Cetus, Emoryville, California) protocol. A second reaction tube contained 400 ng of primers #85 and #94, 200 ng of *Eco* RI/*Bam* HI digested pDZ7, in the same reaction buffer and enzyme, and incubated as above. The products of these reactions were shown to be the correct length as analyzed by gel electrophoresis. A third reaction tube contained 2  $\mu$ l from each product, 400 ng primers #66 and #94 in the same reaction buffer and enzyme, and incubated as above. Primers were designed such that overlap between the two products would allow for PCR synthesis of the combined length fused product. The resultant fragment was extracted with phenol, n-butanol, and ethanol precipitated to remove *Taq* polymerase. The product PCR

fragment was treated with *Bgl* II and *Eco* RI, electrophoresed in low melting agarose, and purified as described above. Meanwhile, the 2.7 kb *Pst* I-*Bgl* II ligase gene containing fragment from pDZ12 and the 2.4 kb *Pst* I-*Eco* RI  $\beta$ -lactamase gene and origin  
5 containing fragment from pFBT64 were purified. All three fragments were combined in a three way ligation and introduced into *E. coli* strain AK53 via transformation. Several clones contained a 5.5 kb plasmid which overproduced ligase under *pho* A promoter control. One such plasmid has been designated pDZ13.

10 In reported studies in overproduction of the thermophilic restriction endonuclease *Taq* I to greater than 30% of soluble *E. coli* proteins [see *Gene* 65:166 (1988)], it was noticed that endonuclease yields were somewhat better if the  $\beta$ -lactamase gene was reversed, and hence transcribing in the opposite  
15 direction as the *pho* A promoter. To make a similar construction with the ligase gene according to the present invention, the 2.3 kb *Pst* I-*Pvu* II fragment from plasmid pFBLT69 (which contains the  $\beta$ -lactamase in reverse orientation) was ligated to the 3.2 kb *Pst* I-*Pvu* II ligase gene containing fragment of plasmid pDZ13.

20 The ligation mix was transformed into *E. coli* strain AK53, and several transformants were analyzed by restriction digests to confirm the orientation of  $\beta$ -lactamase gene. One such clone has been designated pDZ15. Production of ligase in pDZ15 is as good as, if not slightly better than, pDZ13. The ligase enzyme appears  
25 to be somewhat sensitive to proteases, and the cells should be grown for no more than 9 hours after induction. Proteolytic

products of the ligase gene may still have thermostable ligase activity (this has been demonstrated for *Taq* polymerase).

Thermophilic proteins may be substantially modified and still retain sufficient activity for use in the present invention.

5 For example, it has been shown that deletion of approximately one-third of the coding sequence at the amino-terminus of *Taq* polymerase still produces a gene product that is active in polymerase activity [see J. Biol. Chem. 264:6427 (1989)].

Alternatively, another thermophilic protein, the restriction  
10 endonuclease *Taq* I, was shown to retain essentially full activity when amino acids were added to the amino-terminus (+7), the carboxy-terminus (+38), or at certain positions internally (from +2 to +34) [see Gene 65:166 (1988)]. Thus, modification of the primary structure by deletion, n-terminus addition, c-terminus  
15 addition, internal addition or duplication, or alteration of the amino acids incorporated into the sequence during translation can be made without destroying the activity or thermostable nature of the protein. In addition, the availability of DNA encoding these sequences provides the opportunity to modify the codon sequence  
20 so as to generate mutein forms also having ligase activity. Such substitutions or other alterations result in novel proteins having amino acid sequence encoded by DNA falling within the scope of the present invention.

It will also be appreciated that other ligating proteins may  
25 be isolated by the process according to the present invention as exemplified in these examples. Different cell lines may be

expected to produce ligases having different physical properties to that isolated from the *T. aquaticus* HB8 strain used in the making of the present invention. Additionally, variations may exist due to genetic polymorphisms or cell-mediated

5 modifications of the enzyme or its precursors. Furthermore, the amino acid sequence of a ligase so isolated may be modified by genetic techniques to produce ligases with altered biological activities and properties. The resultant DNA sequence may then be able to encode a protein having substantially the same amino  
10 acid sequence as *T. aquaticus* HB8 ligase, but exhibiting a higher or lower level of activity. Such ligating proteins should also be considered to be within the scope of the present invention.

## EXAMPLE VI

(purification of ligase enzyme)

*E. coli* cells AK53 containing plasmids pDZ6 and pGP1-2 (containing the T7 RNA polymerase gene behind the lambda P<sub>L</sub> promoter and under control of the temperature sensitive lambda repressor C1587) [see Proc. Natl. Acad. Sci. USA 82:1074 (1985) and United States Patent 4,795,699], were grown overnight at 32°C on TY plates containing ampicillin at 50 µg/ml and kanamycin at 50 µg/ml to ensure maintenance of both plasmids.

5 Fresh colonies were resuspended in 1 liter of sterile 50 mM Tris HCl buffer at pH 7.6 and containing 6 g NaCl, 25 g Bacto™-tryptone, 7.5 g yeast extract, 1 g glucose, 1.6 g casein amino acid hydrolysate, 50 µg/ml kanamycin and 50 µg/ml ampicillin, and grown at 32°C in a 2 liter flask shaking at 200 rpm. When the

10 O.D.<sub>550</sub> reached between 0.8 and 1.0, synthesis of the T7 polymerase was induced by shifting the cells to 42°C for 30 to 40 minutes. Further synthesis of *E. coli* proteins were inhibited by the addition of 5 ml of 20 mg/ml rifampicin dissolved in methanol to a final concentration of 100 µg/ml. Under these

15 conditions, only genes behind the T7 promoter should be transcribed and hence translated. Cells were incubated for an additional 5 hours at 42°C.

20

Alternatively, *E. coli* cells AK53 containing plasmids pDZ15 (ligase under *pho A* promoter control) were grown

25 overnight at 37°C on TY plates containing ampicillin at 50 µg/ml. Fresh colonies were resuspended in 50 ml of fortified broth

containing 50 µg/ml ampicillin and grown at 37°C in a 500 ml flask shaking at 200 rpm in a G76 benchtop shaker. When the O.D.<sub>500</sub> reached between 0.65 and 0.85, 20 ml was diluted into 1 liter of MOPS media containing 0.2 mM K<sub>2</sub>HPO<sub>4</sub> [see J.

- 5 Bacteriology 119:736 (1974)] to induce the *phoA* promoter. Cells were grown at 37°C in a 2 liter flask shaking at 200 rpm in a G25 floor shaker for an additional 9 hours.

Following incubation, the cells were chilled in ice, harvested by centrifugation (5,000 rpm for 15 min), resuspended  
10 in 20 ml of water, transferred to 35 ml centrifuge tubes, recentrifuged (7,000 rpm for 6 min), and the pellet frozen until ready for protein isolation. After thawing, the pellet was resuspended in 20 ml of buffer A (20 mM Tris HCl buffer at pH 7.6 containing 1 mM EDTA) containing 10 mM 2-mercaptoethanol and  
15 0.15 mM PMSF. After sonication (5 x 1 min at 50% power at 4°C), the solution was centrifuged at 39,000 x g for 60 min.

The enzyme has an estimated molecular weight of from 75,000 to 85,000 daltons when compared with a phosphorylase B standard assigned a molecular weight of 92,500 daltons.

- 20 Alternatively, 2 liters of pDZ15 induced cells were harvested, sonicated, and debris cleared by centrifugation as described above.

The supernatant (40 ml) was brought to 300 mM KCl and passed through a 5 ml DEAE sephacel column to remove  
25 extraneous DNA using 70 ml buffer A containing 0.3 M KCl. The flowthrough fractions containing the ligase were combined, and



treated at 65°C for 20 minutes to irreversibly heat denature many *E. coli* enzymes including endo or exonucleases. Denatured proteins were then removed by centrifugation at 39,000 x g for 15 minutes, and the ligase enzyme precipitated from the supernatant by adding an equal volume of saturated (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> at room temperature for 30 minutes. The ammonium sulfate precipitate was harvested by centrifugation at 8,000 rpm in a clinical centrifuge, and resuspended in 4 ml of distilled water. Samples were dialyzed against buffer A, followed by buffer A containing 50 mM KCl. The dialyzed protein solution was applied to a 40 ml phosphocellulose column equilibrated with buffer A containing 50 mM KCl. After washing with 80 ml of the same buffer, the column was eluted with a 120 ml linear gradient of KCl (0.05 to 0.5 M) in buffer A. The enzyme eluted as a sharper peak from 0.25 to 0.35 M KCl. The protein migrates as two bands of apparent molecular weight approximately 81,000 (adenylated form) and 78,000 (non-adenylated form) and is about 98-99% pure as monitored by SDS-10% polyacrylamide gel electrophoresis. One can convert between the two forms by incubating 150 µg protein in ligase buffer containing either 25 µg nicked Salmon sperm DNA without NAD (resulting in the non-adenylated form), or in ligase buffer with 10 mM NAD (resulting in the adenylated form) for 30 min at 65°C. An equal volume of 20 mM Tris HCl pH 8.0 in 100% glycerol containing 1 mM EDTA, 2 mM dithiothreitol (DTT), and 200 µg/ml Bovine Serum Albumin (Fraction V) is added (final glycerol concentration is 50%), and enzyme stored at either

-70°C or -20°C. From 2 liters of cells, a final yield of 6 mg ligase in 16 ml storage buffer, at 625 nick closing units per microliter. This corresponds to a total of 10,000,000 units of enzyme, and a specific activity of 1,666,667 units/mg.

Since it is known that thermophilic proteins tend to be somewhat more hydrophobic than their mesophilic counterparts, addition of non-ionic detergents or other stabilizing agents may help in long term storage. Storage buffers may therefore include additional components such as glycerol (50%), sucrose (25%), protease inhibitors (0.5-1.0 mM PMSF,  $10^{-7}$  M pepstatin A), salt (KCl, preferably at 100-500 mM), EDTA (0.1-1.0 mM) bovine serum albumin (100-500  $\mu\text{g/ml}$ ), gelatin, dithiothreitol (1-10 mM), and mercaptoethanol (1-10 mM). In addition, it is preferable that the storage buffer contain at least one non-ionic polymeric detergent. A partial listing of such detergents would include ethoxylated fatty alcohol ethers and lauryl ethers, ethoxylated alkyl phenols, polyethylene glycol monooleate compounds, and more particularly Triton X-100, NP-40, and Tween 20 at 0.1-0.5% vol/vol.

To assay for ligase activity, it is important to use a method that is not skewed by the melting temperature ( $T_m$ ) of the substrates. For example, a 4 base cohesive end ligation is most efficient at a low temperature such as  $4^\circ\text{C}$ , well below the temperature optimum for T4 ligase (which is  $37^\circ\text{C}$ ), and certainly below the temperature optimum of a thermophilic ligase. One assay method that should be consistent is the nick-closing assay in which circular plasmid DNA is randomly nicked in several places by DNaseI. The ability of ligase to close all these nicks and generate covalently closed circular DNA can be assayed by separating nicked circle from open circle DNA via electrophoresis in an agarose gel containing ethidium bromide. For example, the

covalently closed circular form of plasmid pUC4KIXX [see Gene  
37:111 (1985)] migrates faster than the linear form, and  
considerably faster than the nicked form on a 1% agarose gel  
containing 0.2 M glycine NaOH pH 8.5 0.1 mM EDTA, and 1 µg/ml  
5 ethidium bromide and run at 150V for 1.5 hr in the same buffer.

## EXAMPLE VII

## (thermophilic ligase assay)

Nicked pUC4KIXX DNA was generated by adding 3  $\mu$ l of freshly diluted 1  $\mu$ g/ml DNaseI to 5  $\mu$ g DNA in 50  $\mu$ l of 50 mM Tris HCl pH 8.0 buffer containing 10 mM  $MgCl_2$ , 1 mM EDTA, and 6 mM mercaptoethanol. The mixture was incubated at room temperature for 5 min, the DNase heat killed at 65°C for 10 min, and the sample stored until used by freezing at -20°C. Under these conditions, about 90% of the DNA was in the nicked circular form, with about 5% in the linear and 5% in the covalently closed circular form.

Thermophilic ligase prepared as above was assayed by adding serial dilutions of ligase to 0.5  $\mu$ g nicked pUC4KIXX in 20  $\mu$ l of 20 mM Tris HCl pH 7.6 buffer containing 50 mM KCl, 10 mM  $MgCl_2$ , 1 mM EDTA, 10 mM NAD, 10 mM dithiothreitol, overlaying with a drop of mineral oil, and incubating at 65°C for 15 min. As a control, T4 ligase was assayed by adding serial dilutions of ligase to 0.5  $\mu$ g nicked pUC4KIXX in 20  $\mu$ l of 50 mM Tris HCl pH 8.0 buffer containing 10 mM  $MgCl_2$ , 1 mM EDTA, 1 mM ATP, 6 mM mercaptoethanol, and incubating at 37°C for 15 min.

Reactions were terminated by the addition of 4  $\mu$ l stop buffer containing 0.2 M EDTA, 50% glycerol, 1% SDS and 0.1% bromphenol blue, and the products were analyzed by gel electrophoresis as described above.

One nick closing unit of ligase is defined as the amount of ligase that circularizes 0.5  $\mu$ g of nicked pUC4KIXX DNA under the buffer and time conditions set forth in the preceding example, such that addition of further ligase does not circularize additional DNA.

As a mini-prep procedure, *E. coli* cells AK53 containing plasmids pDZ15 (ligase under *pho A* promoter control) were grown overnight at 37°C on TY plates containing ampicillin at 50  $\mu$ g/ml. Fresh colonies were resuspended in 5 ml of fortified broth containing 50  $\mu$ g/ml ampicillin, and grown at 37°C. When the O.D.<sub>550</sub> reached between 0.65 and 0.85, 0.12 ml was diluted into 6 ml of MOPS media containing 0.2 mM K<sub>2</sub>HPO<sub>4</sub> to induce the *pho A* promoter. Cells were incubated overnight at 37°C (some proteolysis that occurs after prolonged incubation, so caution is advised in overgrowing induced cells). Cells were harvested in 1.5 ml microcentrifuge tubes, resuspended in 0.3 ml of 20 mM Tris HCL pH 7.6 containing 1 mM EDTA and 10 mM 2-mercaptoethanol, and sonicated 2 x 10 seconds. After clearing debris by centrifugation (12,000 rpm for 2 min.), the supernatant was treated at 65°C for 20 min to irreversibly heat denature many *E. coli* enzymes including the endo and exonucleaseses [see Gene 56:13 (1987)]. The denatured debris was removed by centrifugation and the supernatant assayed as described above. One microliter of this supernatant contained approximately 625 nick closing units of activity.

The *T. aquaticus* ligase preparation described in the preceding examples, as well as commercially available T4 ligase, were shown to contain approximately 125 nick closing units per microliter. Thus, from 1 liter of *E. coli* cells overproducing *T.*  
5 *aquaticus* ligase, the process according to the present invention has purified approximately (800 x 125) 100,000 nick closing units of enzyme.

The thermophilic ligase prepared according to the preceding description has a number of valuable properties which makes it  
10 especially useful as an assay that both amplifies DNA and allows it to discriminate a single base substitution in a DNA sequence. The single most important property of this ligase allowing for these uses is that the ligase retains activity during repeated thermal denaturation/renaturation cycles thus allowing for the  
15 amplification of DNA without necessitating repeated addition of ligase. In addition, the ligase according to the present invention will ligate oligonucleotides of a length which is sufficient to assure their uniqueness in complex genomes at or near the  $T_m$  temperatures of 65°C, and will also accurately discriminate  
20 between exactly complementary and single based mismatched oligonucleotide sequences.

In the simpler of the two procedures developed as a result of cloning the thermophilic ligase DNA sequence, termed a ligase detection reaction (LDR), two oligonucleotide probes are allowed  
25 to hybridize to denatured DNA such that the 3' end of one is immediately adjacent to the 5' end of the other. The

oligonucleotides are chosen to be sufficiently long (20 to 25 nucleotides) such that each will preferentially hybridize to its unique position in the human genome. A thermophilic ligase can then form a covalent phosphodiester bond between the two

5 oligonucleotides, provided that the nucleotides at the junction are perfectly complementary to the target. The specificity of this nick-closing reaction is particularly enhanced by virtue of performing the ligation at or near the  $T_m$  of the two

oligonucleotides for their target. Thus, a single base mismatch at

10 the junction not only forms an imperfect double helix, but also destabilizes the hybrid at the higher temperature. Consequently, thermophilic ligase will efficiently link correctly base paired oligonucleotides and give near zero background ligation in the presence of the imperfectly matched sequences. Using LDR, the

15 amount of product obtained in the ligation reaction can be increased in a linear fashion by repeated thermal cycling.

In the thermophilic ligase chain reaction according to the present invention, both strands serve as targets for oligonucleotide hybridization. By using an additional two

20 oligonucleotides complementary to the opposite strand, the ligation products of one cycle become the targets for the next cycle of ligation as generally depicted in figure 2. For each adjacent oligonucleotide pair, the diagnostic nucleotide is on the 3' side of the junction. Thus, aberrant target independent ligation

25 of complementary oligonucleotides is avoided by use of temperatures near the  $T_m$ , and by taking advantage of the poor



ligation efficiency of single base 3' overhangs. Using ligase chain reaction, the amount of product can be increased in an exponential fashion by repeated thermal cycling.

In order to test the potential of the thermophilic ligase chain reaction (LCR), the gene encoding human  $\beta$  globin was selected as an initial model system to test the technique of the present invention. Previous work has determined that the normal  $\beta^A$  allele and sickle  $\beta^S$  allele differ by a single A->T transversion of the second nucleotide in the sixth codon of the  $\beta$  globin gene, changing a glutamic acid residue into a valine in the hemoglobin  $\beta$  chain according to the following Table I:

TABLE I

Oligonucleotides:		Size	T <sub>m</sub>
#103	GTGTTT - - - - -	27mer	64°C
#102	GTGTT - - - - -	25mer	66°C
#101	CT C - - - - -	23mer	66°C
#107	(5') GACAGC ATG CTG CAC CTG ACT CGT GAG GAG MAG TCT GCG GTT ACT GCG CTG	22mer	70°C
β <sup>A</sup> Globin	CTCTGC TAC CAC CTG CAC TGA GGA CTG CTG TTC ACA CCG CAA TGA CCG GAC		
#109	(3') T - - - - -	22mer	70°C
#104	I - - - - -	24mer	68°C
#105	A - - - - -	26mer	68°C
#106	G - - - - -	28mer	66°C
β <sup>A</sup> Globin	Met Val His Leu Thr Pro Glu Glu Lys Ser Ala Val Thr Ala Leu		
β <sup>S</sup> Globin	Val		

In the following continuation of Table I, presents the oligonucleotide sequences listed in the preceding portion in their conventional 5' --> 3' orientation:

5	Sequence no. (°C)	Sequence 5' --->3'	size (mer)	T <sub>m</sub>
10	101	GT C ATG GTG CAC CTG ACT CCT GA	23	66
	102	GTTT C ATG GTG CAC CTG ACT CCT GT	25	66
	103	GTTTT C ATG GTG CAC CTG ACT CCT GG	27	64
15	104	CT GC AGT AAC GGC AGA CTT CTC CT	24	68
	105	CTTT GC AGT AAC GGC AGA CTT CTC CA	26	68
	106	CTTTTT GC AGT AAC GGC AGA CTT CTC CC	28	66
20	107	G GAG AAG TCT GCC GTT ACT GCC	22	70
	109	C AGG AGT CAG GTG CAC CAT GGT	22	70

Oligonucleotides containing the 3' nucleotide unique to each allele were synthesized with different length 5' tails (see Table I). Upon ligation to the invariant <sup>32</sup>P radiolabelled adjacent oligonucleotide, the individual products could be separated on a polyacrylamide denaturing gel and detected by autoradiography. Based upon these initial findings with autoradiography, subsequent assays were preformed using an automated, non-radioactive detection scheme in which the allele specific oligonucleotides were 5'-biotinylated for capture, and the

invariant oligonucleotides 3'-tailed with digoxigenin. The label was then visualized in an ELISA format using anti-digoxigenin conjugated to alkaline phosphatase, and a colorimetric substrate for the enzyme.

- 5 As depicted in Table I, the nucleotide sequence and corresponding translated sequence of the oligonucleotides used in detecting  $\beta^A$  and  $\beta^S$  globin genes are depicted. Oligonucleotides 101 and 104 detect the  $\beta^A$  target, while 102 and 105 detect the  $\beta^S$  target when ligated to labelled oligonucleotides 107 and 104, respectively. Oligonucleotides 103 and 106 were designed to assay the efficiency of ligation of G:T or G:A and C:A or C:T mismatches using  $\beta^A$  or  $\beta^S$  globin gene targets respectively. Oligonucleotides were designed with slightly different length tails to facilitate discrimination of various products when separated on a denaturing polyacrylamide gel. The tails which were not complementary to the target sequence, may be considered as being "reporter groups" for the individual sequence. Consequently, ligation of oligonucleotides 101, 102, or 103 to 107 gives lengths of 45, 47, or 49 nucleotides, respectively. For the complementary strand, ligation of oligonucleotides 104, 105, or 106 to 109 gives lengths of 46, 48, or 50 nucleotides, respectively. The oligonucleotides were also designed to have calculated  $T_m$  values of 66 to 70°C, which is just at or slightly above the ligation temperature.
- 25 In order to detect the ligation products, oligonucleotides 107 and 109 were 5'-end labelled with  $^{32}P$  using T4

polynucleotide kinase and  $^{32}\text{P}$  according to the following example.

## EXAMPLE VIII

## (radioactive labelling)

Oligonucleotide 107 (0.1  $\mu$ g) was 5' end labelled in 20  $\mu$ l 30 mM Tris HCl buffer at pH 8.0 containing 20 mM Tricine, 10 mM  
5 MgCl<sub>2</sub>, 0.5 mM EDTA, 5 mM dithiothreitol, and 400  $\mu$ Ci of  
[<sup>32</sup>P]ATP, by the addition of 15 units of T4 polynucleotide kinase.  
After incubation at 37°C for 45 min, unlabelled ATP was added to  
1 mM, and incubation was continued an additional 2 min at 37°C.  
The reaction was terminated by the addition of 0.5  $\mu$ l 0.5 M EDTA,  
10 and kinase heat inactivated at 65°C for 10 min. Unincorporated  
<sup>32</sup>P label was removed by chromatography with Sephadex G-25  
pre-equilibrated with TE buffer. Specific activity ranged from 7  
 $\times 10^8$  to  $10 \times 10^8$  cpm/ $\mu$ g of oligonucleotide.

The specificity of the *T. aquaticus* thermophilic ligase according to the present invention for complementary vs. mismatched target was compared under both LDR and LCR conditions (see figure 3 and the following Table II). In the LDR series, two adjacent oligonucleotides were incubated with denatured target DNA and ligase, where the last nucleotide of the unlabelled oligonucleotide was either complemented or mismatched the target DNA. The oligonucleotides were designed with slightly different length tails to facilitate discrimination of various products by allowing them to be separated on a denaturing gel. Consequently, as disclosed earlier, ligation of oligonucleotide 101 ( $\beta^A$  allele), 102 ( $\beta^S$  allele), or 103 to labelled 107 gives lengths of 45, 47 or 49 nucleotides, respectively. For the complementary strand, ligation of oligonucleotides 104 ( $\beta^A$  allele), 105 ( $\beta^S$  allele), or 106 to labelled 109 gives lengths of 46, 48 or 50 nucleotides, respectively. The oligonucleotides were also designed to have a calculated  $T_m$  values of 66°C to 70°C, that is just at or slightly above the ligation temperature. Thus, the specificity of ligating two oligonucleotides hybridized to target DNA with perfect complementarity (A:T) could be directly compared to each possible mismatch (A:A, T:T, G:A, G:T, C:A, or C:T). The methodology for determining specificity of ligation of these oligonucleotides in the presence of  $\beta^A$  or  $\beta^S$  globin gene target was determined as in the following example:

## EXAMPLE IX

(determination of specificity of thermophilic ligase)

Labelled oligonucleotide (200,000 cpm; 0.28 ng; 40 fmoles) and unlabelled oligonucleotide (.27 ng; 40 fmoles) were incubated in the presence of target DNA (1 fmole =  $6 \times 10^8$  molecules *Taq* I digested  $\beta^A$  or  $\beta^S$  globin plasmid) in 10  $\mu$ l 20 mM Tris HCl buffer at pH 7.6 and containing 100 mM KCl, 10 mM  $MgCl_2$ , 1 mM EDTA, 10 mM NAD, 10 mM dithiothreitol, 4  $\mu$ g Salmon sperm DNA, and 15 nick-closing units of the thermophilic ligase, and overlaid with a drop of mineral oil. The reactions were incubated at 94°C for 1 min followed by 65°C for 4 min, and this cycle was repeated between 5 and 30 times. The reactions were terminated by the addition of 8  $\mu$ l formamide containing EDTA (10 mM), xylene cyanol (0.2%), and bromphenol blue (0.2%). Samples (4  $\mu$ l) were denatured by boiling for 3 min prior to loading (40,000 cpm/lane) into the gel.

Products were separated by electrophoresis in which samples were loaded in groups of eight, run into the gel, and then the next set loaded, thereby accounting for the slightly slower mobility of the bands on the right side of the autoradiogram of figure 3. Electrophoresis was in a 10% polyacrylamide gel containing 7 M urea in a buffer of 100 mM Tris borate pH 8.9 and 1 mM EDTA, for 2 hrs at 60 W constant power.

After removing the urea by soaking for 10 min in 10% acetic acid followed by a second soak of 5 min in water, the gels were dried onto Whatman 3 mm paper and autoradiographed overnight at



-70°C on Kodak XAR-5 film (with or without Du Pont Cronex lighting plus intensifying screen). Bands from 20 cycles were excised from the gels and assayed for radioactivity. The results are given in Table II.

TABLE II

Quantitation of complementary and mismatched LDR and LCR bands from 20 cycle LDR and 30 cycle LCR experiments described in Example IX and depicted in Fig. 3 were excised from gels and assayed for radioactivity. Percentage product formed = cpm in product band/cpm in starting oligonucleotide band. Percentage mismatched/complementary = cpm in band of mismatched oligonucleotides/cpm in band of complementary oligonucleotide using the same target DNA, and gives an indication of the noise to signal ratio. LDR amplification was performed using  $6 \times 10^8$  target molecules or 1 femtomole; LCR amplification was performed using  $6 \times 10^6$  target molecules or 10 attomoles.

15	LDR	Oligo base:	Product	mismatched/
		<u>target base</u>	<u>formed (%)</u>	<u>complementary (%)</u>
20		A : T	21.5	
		T : A	13.2	
		T : A	17.9	
		A : T	12.4	
		A : A	<0.1	<0.4
		T : T	0.12	0.7
25		T : T	0.16	1.0
		A : A	<0.1	<0.4
		G : T	0.30	1.4
		C : T	<0.1	<0.4
		G : A	<0.1	<0.4
		C : A	<0.1	<0.4
30	LCR	A:T, T:A	41.4	
		T:A, A:T	10.4	
		A:A, T:T	0.45	1.1
		T:T, A:A	<0.05	<0.2
		G:T, C:A	0.51	1.3
		G:A, C:T	<0.05	<0.2

Thus, the thermophilic *T. aquaticus* ligase was shown to discriminate complementary from mismatched oligonucleotide sequences for all possible mismatched base pairs in LDR assays. Under both competition and individual ligation experiments (at  
5 varying salt concentrations), the worst case mismatch ligations were 1.5 to 1.0% (see Table II, G:T and T:T), while others were 0.4% to <0.1% (see Table II, A:A, C:T, G:A and C:A) of the products formed with complementary base pairs (A:T). This is substantially better than reported (using radioactive detection)  
10 for the mesophilic T4 ligase of *E. coli* [see Gene 76:245 (1989)].

In the LCR amplification/detection series of experiments, two adjacent oligonucleotides were incubated with denatured target DNA and ligase, as well as with the complementary set of oligonucleotides. Under these conditions, the 3' nucleotide of the  
15 unlabelled diagnostic oligonucleotide either complemented or mismatched the target DNA, but always complemented its unlabelled counterpart, i.e. A:T for 101 and 104, T:A for 102 and 105, and G:C for 103 and 106. Thus, an initial "incorrect" ligation of a mismatched oligonucleotide would subsequently be amplified  
20 with the same efficiency as a correct ligation. Samples contained pairs of unlabelled oligonucleotides ( $\beta^A$  allele specific 101 and 104,  $\beta^S$  allele specific 102 and 105, or 103 and 106) with the complementary and adjacent pairs of labelled oligonucleotides, 107 and 109. These labelled and unlabelled  
25 oligonucleotides were incubated in the presence of ligase and 10 attomoles of target DNA (100 fold less target DNA than for LDR)

for 20 or 30 cycles as in Example IX,. The resulting bands are depicted in the left portion of figure 3 and the lower half of Table II.

As can be seen in figure 3 and Table II, the thermophilic  
5 ligase according to the present invention was capable of discriminating complementary from mismatched oligonucleotide sequences for all possible mismatched base pairs in LCR assays. Under both competition and individual ligation experiments the worse case mismatch ligations were from 1.3% to 0.6% (G:T, C:A  
10 and A:A, T:T), while others were <0.2% (T:T, A:A and G:A, C:T) of the products formed with complementary base pairs (A:T, T:A). LCR, using thermophilic ligase according to the present invention, is thus the only method which can both amplify and detect single base mismatches with high signal to noise ratios [see Genomics  
15 4:560 (1989)]. Thus, by utilizing LCR one can detect the difference between a single base mismatch such as occurs between  $\beta^A$  and  $\beta^S$ , and use the results of this assay as a diagnostic for the normal, the carrier, or the diseased patient.

When the entire set of experiments described above were  
20 repeated using buffer containing 150 mM instead of 100 mM KCl, the results were essentially the same as in figure 3 and tabulated in Table II, with ligation of mismatch oligonucleotides for LDR ranging from 0.6% to <0.3% and for LCR ranging from 1.7% to <0.3% of the exactly complementary products.. Thus, the exquisite  
25 discrimination between matched and mismatched oligonucleotides appears not to be critically dependent upon salt conditions.

Alternatively, a different procedure based on phosphatase may also be used. The LCR or LDR reaction may be performed in a 10  $\mu$ l volume under mineral oil. To this is added 50  $\mu$ l of 10 mM Tris HCl pH 7.6 containing 0.5 units of Bacterial Alkaline  
5 Phosphatase (BAP), and 10 mM  $MgCl_2$ , and the incubation continued at 65°C for 2 hrs (note that the ligase enzyme is not killed under these conditions). The 5' end label on an oligonucleotide that has become covalently linked is no longer susceptible to BAP. Ligated product is separated from  
10 monophosphate by the addition of 20  $\mu$ l of 10 mg/ml sonicated salmon sperm DNA as a carrier and precipitated with 20  $\mu$ l of 50% TCA. After centrifugation for 5 min at 12,000 rpm, the supernatant is removed, and the ratio of pellet to pellet + supernatant gives the percentage of product formed. A similar  
15 assay has been used with *Taq* I endonuclease, and the experimental error for positive and negative controls is around 1-2%.

Use of the thermophilic ligase according to the present invention obviates the need to carefully titrate both salt and  
20 enzyme concentration as required for mesophilic ligases. The data from this series of experiments is tabulated in the following Table III.

TABLE III

Quantitation of complementary and mismatched LDR and LCR bands, at 100 and 150 mM KCl concentrations, from 20 cycle LDR and 30 cycle LCR experiments described in Example IX and depicted in Fig. 3. LDR amplification was performed using  $6 \times 10^8$  target molecules or 1 femtomole; LCR amplification was performed using  $6 \times 10^6$  target molecules or 10 attomoles. The mismatched/complementary gives an indication of the noise to signal ratio.

	LDR	Oligo base: target base	Product formed (%)		mismatched/ complementary (%)	
			[KCl] (mM)		[KCl] (mM)	
			100	150	100	150
15		A : T	21.5	23.2		
		T : A	13.2	17.2		
		T : A	17.9	12.8		
20		A : T	12.4	11.7		
		A : A	<0.1	<0.2	<0.4	<0.3
		T : T	0.12	0.21	0.7	0.3
		T : T	0.16	0.30	1.0	0.6
25		A : A	<0.1	<0.2	<0.4	<0.3
		G : T	0.30	0.25	1.4	0.4
		C : T	<0.1	<0.2	<0.4	<0.3
		G : A	<0.1	0.25	<0.4	0.4
		C : A	<0.1	0.20	<0.4	0.3
30	LCR	A:T, T:A	41.4	14.2		
		T:A, A:T	10.4	18.5		
		A:A, T:T	0.45	0.09	1.1	0.6
		T:T, A:A	<0.05	<0.05	<0.2	0.3
		G:T, C:A	0.51	0.24	1.3	1.7
35		G:A, C:T	<0.05	<0.1	<0.2	<0.7

LCR and LDR specificity was tested using both  $\beta^A$  and  $\beta^S$  specific oligonucleotides in direct competition for ligation to the invariant labelled oligonucleotides. Using target DNA ( $\beta^A$ ,  $\beta^S$ , and an equimolar ratio of  $\beta^A$  and  $\beta^S$ ) ranging from 1 femtomole to 1 attomole, thermophilic ligase specifically formed the correct product(s) in each case; no background incorrect ligation product was observed when only one target allele was present). However, the efficiency of forming the  $\beta^S$  specific products is somewhat less than forming the  $\beta^A$  products, and after 20 cycles of amplification, the  $\beta^S$  specific products were approximately one-third of the  $\beta^A$  specific products as quantitated by assaying excised products for radioactivity. Hence a direct competition assay, wherein two oligonucleotides are differentially labelled (for example with fluorescent groups) to quantitate the relative initial concentrations of each target sequence allele will require careful titrations for each allele.

The specificity of LCR DNA amplification with sub-attomole quantities of target DNA was also examined. The extent of LCR DNA amplification was determined in the presence of target DNA ranging from 100 attomoles ( $6 \times 10^7$  molecules) to less than one molecule per tube. Reactions were incubated for 20 or 30 cycles, and products separated and quantitated as depicted in figure 4 and the following table IV.

TABLE IV

- 5 Quantitation of LCR amplification. Bands from 30 cycle LCR experiments were excised from the gels and assayed for radioactivity. At higher target concentration, DNA amplification was essentially complete after 20 cycles; slightly imprecise excision of 30 cycle bands from this portion of the gel probably accounts for product formed values in excess of 100%.
- 10 Percentage product formed = cpm in product band/cpm in starting oligonucleotide band; Amplification = No. of product molecules formed/No. of target molecules

	Target Molecules	Product formed (%)	Amplification _____
15	$6 \times 10^7$	134	
	$2 \times 10^7$	96	
	$6 \times 10^6$	107	
	$2 \times 10^6$	78	
20	$6 \times 10^5$	85	
	$2 \times 10^5$	48	$5.8 \times 10^4$
	$6 \times 10^4$	25	$1.0 \times 10^5$
	$2 \times 10^4$	4.5	$5.4 \times 10^4$
	$6 \times 10^3$	2.3	$9.2 \times 10^4$
25	$2 \times 10^3$	0.36	$4.3 \times 10^4$
	$6 \times 10^2$	0.18	$7.2 \times 10^4$
	$2 \times 10^2$	0.14	$1.7 \times 10^5$
	60	<0.05	
	20	<0.05	
30	6	<0.05	
	2	<0.05	
	0	<0.05	

In the absence of target, no background signal was detected

35 when carrier salmon sperm DNA (4  $\mu$ g) was present as seen in



figure 4. At higher initial target concentrations, DNA amplification was essentially complete after 20 cycles, while at lower initial target concentrations substantially more product is formed with additional amplification cycles. Under these  
5 conditions, 200 molecules of initial target DNA could easily be detected after 30 cycles.

The thermostable nature of the enzyme is readily apparent in figure 4. By comparing the amount of product formed after 20 cycles to that formed after 30 cycles, it is apparent that at the  
10 lower target DNA concentrations additional product is formed after more cycles (see especially  $2 \times 10^4$  to  $2 \times 10^2$  target DNA molecules). In other words, the enzyme still has activity after 20 cycles of 94°C for 1 minute followed by 65°C for 4 minutes.

Thus, *T. aquaticus* ligase retains the ability to catalyze  
15 formation of a phosphodiester bond between two adjacent oligonucleotides hybridized to a complementary strand of DNA at a temperature in the range of about 50°C to about 85°C after repeated exposure to temperatures that denature DNA, namely in the range of about 105°C for about 0.25 minutes to about 4  
20 minutes.

Hence, the specific amplification of a nucleic acid test substance of known nucleotide sequence using LCR requires: (1) two adjacent oligonucleotides complementary to and in molar excess of the target sequence nucleic acid, and having no  
25 mismatch to the target sequence nucleic acid at the junction of the adjacent oligonucleotides; (2) a second set of adjacent

oligonucleotides complementary to the first set of adjacent oligonucleotides, complementary to and in molar excess of the target sequence nucleic acid, and having no mismatch to the target sequence nucleic acid at the junction of this second set of adjacent oligonucleotides; (3) a thermostable ligase which does not become irreversibly denatured and lose its catalytic ability when subjected to temperatures of from about 50°C to about 105°C; and (4) subjecting this ligase mixture to repeated temperature cycles which comprises a first temperature to denature the DNA (in a range of about 90°C to about 105°C), and a second temperature to allow for hybridization/ligation (in a range of about 50°C to about 85°C). In the amplification of  $\beta^A$  globin allele described above, the components were (1) oligonucleotides 101 and 107; (2) oligonucleotides 104 and 109; (3) *T. aquaticus* ligase; and (4) 30 temperature cycles of 94°C for 1 minute followed by 65°C for 4 minutes.

In figure 4, bands of 45 and 46 nucleotides correspond to ligation products of the coding and complementary  $\beta^A$  globin oligonucleotides. Lower molecular weight products correspond to ligation of deletion oligonucleotides present in the initial ligation reaction. Since samples were loaded in groups of eight, the right side of the autoradiogram gives the appearance of slower migration.

To further test the ability of ligase to discriminate between complementary and mismatched oligonucleotides, an LCR experiment was performed in the presence and absence of

oligonucleotides which would give G-T and C-A mismatches in accordance with the following example which not only shows DNA amplification, but also supports the thermostable nature of the enzyme found in Example IX.

## EXAMPLE X

One set of experiments contained 40 fmoles each of unlabelled 101 and 104 oligonucleotides, while the second set had in addition 40 fmoles of unlabelled 103 and 106 oligonucleotides.

5 Both sets contained 40 fmoles each of labelled 107 and 109.

Labelled oligonucleotides (200,000 cpm; .28 ng; 40 fmoles) and unlabelled oligonucleotides (.27 ng; 40 fmoles) were incubated in the presence of target DNA, ranging from 100 attomoles ( $6 \times 10^7$  molecules) to 0.01 attomoles ( $6 \times 10^3$  molecules) of *Taq* I

10 digested  $\beta^A$  or  $\beta^S$  globin plasmid. Incubation was carried out in 10  $\mu$ l 20 mM Tris-HCl, pH 7.6 buffer containing 100 mM  $MgCl_2$ , 1 mM EDTA, 10 mM NAD, 10 mM dithiothreitol, 4  $\mu$ g Salmon sperm DNA, and 15 nick-closing units of *T. aquaticus* ligase, and overlaid with a drop of mineral oil. Reactions were incubated at 15 94°C for 1 min followed by 65°C for 4 min, and this cycle was repeated 20 or 30 times.

The resulting samples were electrophoresed, gel autoradiographed overnight with the aid of a Cronex intensifying screen and the bands counted. The bands from the 20 autoradiographed gel are depicted in figure 4, and the quantitation of LCR amplification tabulated in the following Table V.

TABLE V

Quantitation of LCR amplification the presence or absence of mismatched competitor molecules.

	Target molecules	Complementary Oligonucleotides (101, 104) (A:T, T:A)		Complementary & Mismatched Oligonucleotides (101, 104 & 103, 106) (A:T, T:A & G:T, C:A)		
		Product formed	Amplification	Product formed	Amplification	Mismatched/Complementary
5						
10	$6 \times 10^7$ ( $\beta^A$ )	114		93		1.0
	$2 \times 10^7$	93		95		1.8
	$6 \times 10^6$	102		93		0.5
	$2 \times 10^6$	90		67		0.5
15	$6 \times 10^5$	51		46		
	$2 \times 10^5$	31	$3.7 \times 10^4$	23	$2.8 \times 10^4$	
	$6 \times 10^4$	17	$6.8 \times 10^4$	9.3	$3.7 \times 10^4$	
	$2 \times 10^4$	8.6	$1.0 \times 10^5$	2.9	$3.5 \times 10^4$	
	$6 \times 10^3$	3.2	$1.3 \times 10^5$	0.8	$3.4 \times 10^4$	
20	0	<0.1		<0.1		
	$6 \times 10^7$ ( $\beta^S$ )	2.1		1.5		

At high target concentrations, sufficient mismatched product was produced to be visualized (as in figure 4), the quantity of mismatched product ranging from 1.8% to 0.5% of the complementary product. Use of an excess of mismatched target DNA ( $\beta^S$  instead of  $\beta^A$  globin DNA at  $6 \times 10^7$  molecules per tube) gave only 2.1% and 1.5% product. The same amount of product may be formed when using three to ten thousand fold less complementary target DNA. Based upon this, the signal from correctly paired ligation products is 50 to 500 fold higher than

mismatched products under competition or individual LCR ligation conditions.

At low target concentrations, the extent of DNA amplification ranged from  $3.7 \times 10^4$  to  $1.7 \times 10^5$  (see Tables IV and V). Assuming the efficiency of ligation is the same in each cycle, the average amplification per cycle is between 40 and 50%.

The efficiency per cycle could, of course, be potentially enhanced by altering buffer conditions, enzyme concentration, or thermal cycling times and temperatures - all within the capabilities of those skilled in the art. It has, for example, been shown that the ligation efficiency of thermophilic ligase (and other ligases) may be enhanced by altering buffer compositions, such as using  $\text{NH}_4\text{Cl}$ , HEPES, polyamines such as spermidine, or polyethylene glycols [see J. Biol. Chem 259:10041 (1984), and J. Biochem. 100:123 (1986)]. Varying the amounts of each component in the currently used buffer and either supplementing or exchanging one or more components with, but not limited to, the chemical and biological components listed above, are among the methods of improving LCR that are straight forward for those skilled in the art. One skilled in the art can also easily vary the cycling times and temperatures. For example, at later time points, the majority of target present is oligonucleotide product from a previous LCR reaction. These oligonucleotides are short (preferably but not limited to 40-60 mers) and may melt more rapidly, allowing more rapid cycling. In the present invention, successful ligase chain reactions have been completed for 30 and

40 cycles under cycling conditions of 94°C for 0.5 minutes followed by 65°C for 2 minutes (half the time of the 1 minute at 94°C and 4 minutes at 65°C cycle time for the preferred ligase chain reaction conditions). Both the ligation temperature and the DNA denaturing temperatures may be varied with respect to actual degree, duration, and number of repeated cycles. Optimal conditions must maximize the amount of product formed in the presence of perfectly complementary target DNA, while minimizing the amount of incorrect product formed in the presence of mismatched target DNA or in the absence of complementary target DNA.

Utilizing these findings, a method for the detection of specific sequences of oligonucleotides in clinical samples was developed. The source of the sample may be any material or substance which comprises nucleic acid. The nucleic acid need not be a naturally occurring nucleic acid, but may be synthesized by chemical, enzymatic, or biological means and may have other than naturally occurring purines and pyrimidines. The source of the clinical sample may be cellular or non-cellular, and may be derived from such physiological media as blood, serum, plasma, breast milk, stool, pus, tissue scrapings, washings, urine, or the like. Furthermore, the sample may be associated with a set or subset of cells, such as neoplastic cells, lymphocytes (for example, T-cells or B-cells, monocytes, neutrophils, etc); may include pathogens including viruses, bacteria, mycoplasma, fungi, protozoa, etc.; may include constructs, etc. or RNA, such as

5 messenger RNA, transfer RNA, ribosomal RNA, viruses, or the like;  
and it may involve structural genes, untranslated regions,  
regulatory regions, introns, exons, or the like. In addition, the  
detection may be for a wide variety of purposes such as, for  
example, the diagnosis of a potential or actual disease state in  
10 plant or animal species, as well as the detection of sets or  
subsets of pathogens, the monitoring of genetic engineering, or  
the like.

One such method for which the present invention may be  
10 used (and which clearly demonstrates the feasibility of direct  
LCR allelic detection from blood samples without the need for  
prior PCR amplification) is embodied, for example, in the  
detection of  $\beta$ -globin alleles in human genomic DNA. Based upon  
the high level of DNA amplification, the allele specific LCR  
15 detection of DNA was examined from blood collected from normal  
( $\beta^A\beta^A$ ), carrier ( $\beta^A\beta^S$ ), and sickle cell ( $\beta^S\beta^S$ ) individuals as  
more fully described in the following example:



## EXAMPLE XI

(detection of  $\beta$ -globin alleles in human genomic DNA)

Human genomic DNA was isolated from 0.5 ml whole blood [see *PCR Technology*, H. A. Erlich editor, Stockton Press (1989) pg 36]. Whole blood (0.5 ml) was mixed with an equal volume of lysis buffer (10 mM Tris-HCl, pH 7.6, containing 5 mM  $MgCl_2$  and 0.32 M sucrose). After a brief centrifugation (1 min at 12,000 rpm in an eppendorf desktop centrifuge), the supernatant was very carefully removed, leaving 0.15 to 0.2 ml of supernatant and loosely pelleted nuclei. The pellet was resuspended with vortexing in an additional 0.5 ml lysis buffer, nuclei pelleted and the supernatant removed as above. This step was repeated three or four times until the supernatant was clear or just barely pink. After removal of the final supernatant (again leaving about 0.15 to 0.2 ml), 0.25 ml of LCR DNA Buffer containing non-ionic detergents (20 mM Tris-HCl, pH 7.6, containing 2 mM EDTA and 0.45% each of non-ionic detergents NP40 and Tween 20) was added. Any excess RNA was digested by the addition of 2  $\mu$ l of 4 mg/ml heat treated RNase A for 15 min at 37°C. Any proteins were digested by the addition of 5  $\mu$ l of 10 mg/ml freshly made Proteinase K and incubation at 50°C for 1 to 2 hours. Proteinase K and RNase A were removed by sequential extractions with phenol, phenol/chloroform, chloroform, n-butanol (2X) and the nucleic acid recovered by precipitation with ethanol. Samples were boiled for 5 min prior to use in LCR assays.

Each isolated human genomic DNA was tested in two reaction mixtures, the first testing for the presence of the normal  $\beta^A$  allele, and the second testing for the presence of the sickle  $\beta^S$  allele. The first reaction mixture contained  $\beta^A$  test  
5 oligonucleotides 101 and 104 (0.27 ng or 40 fmoles each), labelled oligonucleotides (107 and 109; 200,000 cpm (0.28 ng or 40 fmoles each), genomic DNA (corresponding to 10  $\mu$ l of blood, or about  $6 \times 10^4$  nucleated cells) in 10  $\mu$ l 20 mM Tris-HCl buffer, pH 7.6, containing 100 mM KCl, 10 mM  $MgCl_2$ , 1 mM EDTA, 10 mM NAD,  
10 10 mM dithiothreitol, and 15 nick-closing units of *T. aquaticus* ligase, and overlaid with a drop of mineral oil. The second reaction mixture contained  $\beta^S$  test oligonucleotides 102 and 105 (0.27 ng or 40 fmoles each), labelled oligonucleotides 107 and 109 (200,000 cpm or 0.28 ng or 40 fmoles each), genomic DNA  
15 corresponding to 10  $\mu$ l of blood or about  $6 \times 10^4$  nucleated cells) in 10  $\mu$ l 20 mM Tris-HCl buffer, pH 7.6 and containing 100 mM KCl, 10 mM  $MgCl_2$ , 1 mM EDTA, 10 mM NAD, 10 mM dithiothreitol, and 15 nick-closing units of *T. aquaticus* ligase, and overlaid with a drop of mineral oil.

20 Both reaction mixtures were incubated at 94°C for 1 min followed by 65°C for 4 min, and this cycle was repeated 20 to 30 times. Reactions were terminated by the addition of 8  $\mu$ l formamide containing EDTA (10 mM), xylene cyanol (0.2%), and bromphenol blue (0.2%).

25 Samples (4  $\mu$ l) were denatured by boiling for three min prior to loading (40,000 cpm/lane). Electrophoresis was in a 10%

polyacrylamide gel containing 7 M urea in a buffer of 100 mM Tris borate at pH 8.9 and 1 mM EDTA, for 2 hours at 60 Watt constant power. After removing the urea (10 min soak in 10% acetic acid, followed by 5 min soak in H<sub>2</sub>O). Gels were then dried onto

- 5 Whatman 3 mm paper and autoradiographed overnight at -70°C on Kodak XAR-5 film with a DuPont Cronex intensifying screen. Ligation products of 45 and 46, or 47 and 48 nucleotides indicate the presence of the  $\beta^A$  or  $\beta^S$  globin gene, respectively. As noted with plasmid derived target DNA, the efficiency of ligation (and
- 10 hence detection) is somewhat less for the  $\beta^S$  than the  $\beta^A$  specific oligonucleotides.

Figure 5 is an autoradiogram showing the detection of  $\beta$ -globin alleles in human genomic DNA made in accordance with the proceeding example. Ligation products of 45 and 46, or 47 and 48 nucleotides indicate the presence of the  $\beta^A$  or  $\beta^S$  globin gene, respectively. Thus, with target DNA corresponding to 10  $\mu$ l of blood,  $\beta^A$  and  $\beta^S$  alleles could be readily detected using allele specific LCR.

Hence, the successful detection of a biologically derived nucleic acid test substance, which has a known normal nucleotide sequence and a known possible mutation at at least one target nucleotide position in the sequence, requires (1) a first reaction mixture comprising two sets of adjacent oligonucleotides complementary to each other, complementary to the target sequence nucleic acid, wherein there is at least one mismatched base pair to the mutant target sequence nucleic acid, but not to the normal target sequence nucleic acid at the junction of the adjacent oligonucleotides; (2) a second reaction mixture comprising two sets of adjacent oligonucleotides complementary to each other, complementary to the target sequence nucleic acid, wherein there is at least one mismatched base pair to the normal target sequence DNA, but not to the mutant target sequence nucleic acid at the junction of the adjacent oligonucleotides; (3) a thermostable ligase which does not become irreversibly denatured and lose its catalytic ability when subjected to temperatures of from about 50°C to about 105°C; and (4) subjecting these ligase mixtures to repeated temperature cycle

which comprises a first temperature to denature the DNA (in a range of about 90°C to about 105°C), and a second temperature to allow for hybridization/ligation (in the range of about 50°C to about 85°C) - this also allows adjacent oligonucleotides in each reaction mixture to become possibly covalently linked; (5) separating the test substance and any unlinked test oligonucleotides from covalently linked oligonucleotide product (if formed); and (6) detecting the presence or absence of covalently linked oligonucleotides in each reaction mixture whereby the presence of covalently linked oligonucleotide product in the first reaction mixture indicates the presence of normal target sequence and the presence of covalently linked oligonucleotide product in the second reaction mixture indicates the presence of mutant target sequence. In the detection of  $\beta^A$  and  $\beta^S$  globin alleles described above, the components were (1) oligonucleotides 101, 104, 107 and 109; (2) oligonucleotides 102, 105, 107 and 109; (3) *T. aquaticus* ligase; (4) 30 temperature cycles of 94°C for 1 min followed by 65°C for 4 min; (5) denaturing nucleic acids by boiling in 45% formamide and separating on a sequencing gel; and (6) autoradiographing of the gel.

This clearly demonstrates the feasibility of direct LCR allelic detection from blood samples according to the present invention without the need for PCR amplification.

As noted with plasmid derived target DNA, the efficiency of ligation (and hence detection) is somewhat less for the  $\beta^S$  than

the  $\beta^A$  specific oligonucleotides. After 30 cycles of amplification,  $\beta^S$  specific products were approximately one-third of  $\beta^A$  specific products, as quantitated by assaying excised products for radioactivity. These differences may be a function of the exact nucleotide sequence at the ligation junction, or the particular oligonucleotides (with differing 5' tails) used in the LCR experiments. However, the present invention still allows for a direct competition assay where two oligonucleotides are differentially labelled (for example with fluorescent groups or, in this case, with different length tails) to determine the presence or absence of either allele in a reaction mixture. In the generalized form, the method according to the present invention allows one to assay two alleles in the same vessel, providing the sets of oligonucleotides containing at least one mismatched base pair to the mutant target sequence nucleic acid, but not to the normal target sequence nucleic acid at the junction of the adjacent oligonucleotides, are labelled with one set of labels, and the oligonucleotides containing at least one mismatched base pair to the normal target sequence nucleic acid, but not to the mutant target sequence nucleic acid at the junction of the adjacent oligonucleotides, are labelled with a different label.

In a comparable non-radioactive assay, as depicted in figure 6, a minimum of two oligonucleotide probes are synthesized and modified for particular functions in the ligation assay. One probe contains a hook that permits the capture of the oligonucleotide following ligation. An example of such a hook is biotin which can

be captured by streptavidin or avidin bound to appropriate supports. The other probe has a reporter group. Although a variety of reporter groups, both radioisotopic and non-radioactive, are available and can be used with the assay according to the present invention, such as fluorophores or luminescent moieties, the currently preferred reporter is one which may participate in an ELISA (enzyme-linked immunosorbent assay). More specifically, figure 6 depicts a schematic diagram of an ELISA based oligonucleotide ligation assay in which biotinylated (B) and digoxigenin-labelled (D) oligonucleotides are hybridized with a DNA target in the presence of ligase (arrow). Biotinylated oligonucleotides are captured on streptavidin (SA) coated within the wells of microtiter plates. The wells are washed to remove unbound oligonucleotides, and alkaline phosphatase (AP) conjugated anti-digoxigenin antibodies (D) are added to the wells. Following an incubation and wash cycle, alkaline phosphatase substrate (S) is added, and digoxigenin detected by the production of a color product.

The non-radiolabelled assay according to the present invention consists of several steps: (1) preparation of the DNA target; (2) denaturation and hybridization of the modified oligonucleotide probes; (3) ligation; (4) capture of the biotinylated probe; (5) washing to remove free nonbiotinylated oligonucleotides and target; (6) addition of alkaline phosphatase conjugated anti-digoxigenin antibodies; (7) washing to removed unbound antibody; (8) addition of alkaline phosphatase substrate;

and (9) spectrophotometric analysis. The following flow chart details the general procedure (which has automated on a modified Biomek 1000 workstation instrument) by which a non-radiolabelled assay according to the present invention can be

5 conducted:



## Amplified Target DNA

## T4 Ligase Detection

## Taq Ligase Detection

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Denature residual *Taq* polymerase by adding:10 45  $\mu$ l of 0.3 N NaOH45  $\mu$ l of 0.1 N KOH

Renature target DNA by adding:

15

45  $\mu$ l of 0.3 N HCl45  $\mu$ l of 0.1 N HCl

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Distribute amplified target to microtiter plates at 10  $\mu$ l per wellAdd biotinylated and reporter oligonucleotides to DNA targets  
(200 fmoles of each oligonucleotide in 10  $\mu$ l of 2 X ligation mix)

25

Ligation Mix:  
 200 fmole biotinylated oligo  
 200 fmole reporter oligo  
 100 mM Tris-HCl, pH 7.5  
 20 mM MgCl<sub>2</sub>  
 10 mM DTT  
 2 mM ATP  
 2 mM Spermidine  
 50% Formamide

30

10 mM DTT  
 2 mM ATP  
 2 mM Spermidine  
 50% Formamide

35

Ligation Mix:  
 200 fmole biotinylated oligo  
 200 fmole reporter oligo  
 100 mM Tris-HCl, pH 7.5  
 20 mM MgCl<sub>2</sub>  
 10 mM DTT  
 2 mM ATP  
 2 mM Spermidine  
 2 mM NAD  
 100 mM KCl  
*Taq* Ligase

Denature target oligonucleotide mix at 93°C for 2 minutes

40

Cool to room temperature and add 5  $\mu$ l T4 ligase in  
 200 mM NaCl  
 50 mM Tris-HCl, pH 7.5  
 10 mM MgCl<sub>2</sub>  
 5 mM DTT  
 1 mM ATP  
 1 mM Spermidine

45

5 mM DTT  
 1 mM ATP  
 1 mM Spermidine

Cool to 60-68°C and ligate  
 for 15 minutes (repeat  
 denaturation and ligation  
 step to amplify)

50

Ligate at room temperature (25°C) for 15 minutes

Stop ligation reaction and denature products by adding 10  $\mu$ l of 0.3 N NaOH  
Neutralize reactions by adding 4  $\mu$ l of 3 M sodium acetate

- 5            Transfer reactions to an avidin coated and blocked microtiter plate  
(avidin coating - 60  $\mu$ g of avidin/well in 60  $\mu$ l of PBS, pH 7.0 for 60 min at 37°C;  
blocking - remove avidin from the plate and add 200  $\mu$ l/well of 100 mM Tris-HCl,  
150 mM NaCl, 0.05% Tween, 0.5% dry milk, and 100  $\mu$ g/ml of salmon sperm DNA)
- 10           Capture biotinylated oligonucleotide at room temperature for 30 minutes
- 15           Wash plate to remove unbound oligonucleotides and targets with (1) 100 mM Tris-  
HCl, pH 7.5, in 150 mM NaCl in 0.05% Tween; (2) 0.01 N NaOH in 0.05% Tween;  
and (3) 100 mM TRIS-HCl, pH 7.5 in 150 mM NaCl in 0.05% Tween
- 20           Add alkaline phosphatase conjugated antibody to the reporter oligonucleotide; 30  $\mu$ l per  
well in 100 mM TRIS-HCl, pH 7.5, 150 mM NaCl, 0.5% dry milk and 0.05% Tween
- Incubate plates for 30 min at room temperature for antibody binding to the reporter
- 25           Wash the plate with 100 mM TRIS-HCl, pH 7.5, 150 mM NaCl in 0.05% Tween to  
remove unbound antibody
- 30           Add substrate
- Read plate for appropriate colormetric, chemiluminescent, or fluorescent product

Genomic sequences required to begin this assay can be amplified by a number of different methods, including LCR, 3SR, and PCR. We have used PCR amplification to obtain DNA targets listed on the following Table VI for litigation assay primers:

TABLE VI

(sequences of amplification primer sets)

<u>Target Gene</u>	<u>Amplification Primers</u>
B-globin	CAACTTCATCCACGTTACCTTGCC AGGGCAGGAGCCAGGGCTGGGG
alpha <sub>1</sub> -antitrypsin	TCAGCCTTACAACGTGTCTCTGCTT GTATGGCCTCTAAAAACATGGCCCC
cystic fibrosis	CAGTGGAAGAATGGCATTCTGTT GGCATGCTTTGATGACGCTTCTG

15

DNA amplification was performed using 5  $\mu$ l of DNA (2 ng/ $\mu$ l for genomic DNA or 5  $\mu$ l of treated material from an alternative source) is mixed with a pair of primer oligonucleotides (0.5  $\mu$ M each) specific for the region of DNA to be amplified in a PCR buffer containing 0.05 U/ $\mu$ l of Taq polymerase, 50 mM KCl, 25 mM Tris HCl buffer at pH 8.3, 10 mM MgCl<sub>2</sub>, 200  $\mu$ g/ml gelatin, 0.1% Triton X-100, and 1.5 mM each of dATP, dCTP, dGTP and dTTP. The sample was overlaid with 60  $\mu$ l of light mineral oil, denatured at 93°C for 5 min target, and subjected to 40 cycles consisting of 20 sec at 93°C, 40 sec at 55°C, and 1 min at 72°C. Following temperature cycling, the

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sample was subjected to 10 min at 72°C to complete extension of the DNA sample.

Oligonucleotides are synthesized and modified for particular functions in the ligation assay. The assay requires a minimum of two modified oligonucleotides. One oligonucleotide has a hook that permits capture of the oligonucleotide following ligation. An example of this is a biotinylated oligonucleotide which can be captured on streptavidin or avidin supports. The other oligonucleotide has a reporter group which, in the case of a fluorophore reporter, multiple reporters with different emission spectra could easily be incorporated into a single assay.

For an ELISA based system, probes which discriminate allelic forms of a gene are synthesized with a 5' biotin group. Reporter probes are enzymatically or chemically 5'-phosphorylated and labelled with the hapten digoxigenin. The hapten is added to the 3' end of the reporter probe by tailing 500 pM of oligonucleotide at 37°C for 1 hour in 10 mM potassium cacodylate, pH 7.0, 1 mM CoCl<sub>2</sub>, 0.1 mM DTT, 5 nM of digoxigenin dUTP, 0.05 μM of dATP, and 100 units of the enzyme terminal transferase in a total volume of 20 μl. After labelling, 2 μl of 3 M sodium acetate and 1 μl of yeast t-RNA (1 mg/ml) and 60 μl of 95% ethanol is added. The oligonucleotide is precipitated at 4°C for 5 min and then collected by centrifugation at 6500 x g for 5 minutes. The pellet is resuspended in 20 μl of distilled water and the process repeated. This precipitation removes unconjugated excess digoxigenin from the labelled probe. Example of

oligonucleotides which discriminate alleles for three pathologic states are given in the following table VII:

TABLE VII

(sequences of example oligonucleotides for ELISA detection)

5				
	Target Gene	Form of Gene Detected	Biotinylated Primer	Labelled (L) Primer
10	$\beta$ -globin	$\beta^A$	B1-ATGGTGCACTGACTCCTGA	GGAGAAGTCTGCOGTACTG
		$\beta^S$	B2-ATGGTGCACTGACTCCTGT	
	$\alpha_1$ anti-trypsin	M	B1-GGCTGTGCTGACCATGACG	AGAAAGGGACTGAAGCTGCT
		Z	B2-GGCTGTGCTGACCATGACA	
15	cystic fibrosis	non-508	B1-ATTAAAGAAAATATCATCTT	TGGTGTTTCCTATGATGAAT
		508	B2-ACCATTAAAGAAAATATCAT	

Utilizing the procedure contained in the previous flow chart,  
 20 a number of experiments were run and, after color development,  
 data were obtained spectrometrically at a wavelength of 490 mN.  
 Typical results for such tests have been tabulated in the  
 following table VIII:

TABLE VIII

(spectrophotometric data from automated ligation reactions using Taq ligase)

Amplified Genomic DNA Target From:	<u>Ligation Primer Mix</u>	
	B1 + L	B2 + L
10 B - globin		
$\beta^A$	$1.27 \pm 0.06$	$0.01 \pm 0.01$
$\beta^S$	$0.04 \pm 0.03$	$1.85 \pm 0.03$
alpha <sub>1</sub> -antitrypsin		
M	$1.85 \pm 0.15$	$0.03 \pm 0.01$
15      Z	$0.03 \pm 0.03$	$1.47 \pm 0.07$
cystic fibrosis:		
non-508	$1.33 \pm 0.20$	$0.02 \pm 0.01$
508	$0.01 \pm 0.01$	$1.66 \pm 0.16$

20      Comparable levels of detection were achieved with either T4 or Taq ligase. In addition, a number of ligation reactions have been performed for several other disease associated polymorphisms with comparable results. Additionally, eight different polymorphisms in the human T cell receptor loci have

25      been examined with similar detection results. The present invention, therefore, appears to be generally applicable in the analysis of DNA polymorphisms consisting of single base substitutions, DNA deletion or insertions, or DNA translations.

30      In addition, a number of alkaline phosphatase substrates can be employed in the ELISA assay of the present invention including

sensitive chemiluminescent substrates (10 attomole detection). The format of the assay is easily adapted to other reporter formats such as fluoropores which can be read in the appropriate microtiter format. Incorporation of the appropriate fluorophore format would, for example, permit multiplex analysis by ligation. In this scheme, oligonucleotides discriminating different alleles and/or different genes could be evaluated in a single assay.

Furthermore, it is also possible that tandem ligation assays (ligation of oligonucleotides in chains) could be employed to assess closely spaced DNA polymorphisms such as those which exist in the major histocompatibility complex genes. Such modifications to the assay specifically depicted above are considered to be well within the scope of the present invention

The present invention can be used in a wide variety of DNA diagnostic screening. For example, and not intending to limit the scope of the present invention, such DNA diagnostic screens may include those according to the following summary:

#### A - INFECTIOUS DISEASES:

1. Viral Diseases: HIV, EBV, HPV, HSV, CMV, Hepatitis (non-A, non-B)
  - (i) blood and tissue screening
  - (ii) rapid identification
  - (iii) distinguish chronic infection from past exposure
  - (iv) distinguish resistant strains in mixed infection
2. Bacterial Diseases: Mycobacteria, Syphilis, Chlamydia, Legionella, Campylobacter, Pneumocystis, Listeria, Lyme, Leprosy
  - (i) rapid identification of slow growing microbes

(ii) identification in immuno-deficient patients

(iii) testing food for contamination

3. Parasitic Diseases: Malaria, Trypanosomes, Leishmania

(i) rapid identification of "third world" blood diseases

(ii) screening travelers and armed forces

B - GENETIC DISEASES:

1. Single Allele Diseases: Cystic Fibrosis, Duchenne's muscular dystrophy, Sickle Cell Anemia,  $\beta$ -thalasemia, Haemophilia A, Gaucher, Tay-Sachs, Alzheimer's, Neurofibromatosis

2. Cancer: Retinoblastoma, Wilms tumor, Colon, Breast, Oncogenes, Tumor suppressors

3. Multiple Allele Diseases: Coronary heart disease, Diabetes, High blood pressure, Schizophrenia, Manic-depression, Alcohol abuse

(i) predisposition to disease

(ii) preventive medicine, exercise, diet

(iii) genetic screening and counseling

(iv) gene therapy.

C - GENETIC IDENTIFICATION:

1. Humans: HLA typing, forensics

(i) tissue transplantation

(ii) genetic linkage analysis

(iii) human genome program

(iv) positive identification of missing children

2. Animals: Horses, Dairy cows, Cattle, Domestic pets

(i) pure genetic characteristics

(ii) confirm breeding lineage

(iii) positive identification of animals

3. Plants: Seed Stock

(i) assure genetic diversity

(ii) identify strains resistant to drought and disease



Thus, while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we therefore do not wish to be limited to the precise terms set forth, but desire to avail ourselves of such changes and alterations which may be made for adapting the invention to various usages and conditions. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents, and therefore within the purview of the following claims.

Having thus described our invention and the manner and a process of making and using it in such full, clear, concise and exact terms so as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same;

## WE CLAIM:

1. The cell line AK76 designated as ATCC 55032.
2. A plasmid selected from pDZ1 and designated as ATCC 68307, and pDZ7 and designated as ATCC 68308.
3. A purified isolated DNA fragment or nucleic acid sequence that hybridizes to such sequence under high stringency consisting essentially of a DNA sequence coding for a thermostable *Thermus aquaticus* strain HB8 ligase enzyme.
4. A purified isolated DNA fragment or nucleic acid sequence that hybridizes to such fragment under high stringency which comprises the partial sequence coding for a thermostable ligase having the nucleic acid sequence

ATG ACC CTG GAA GAG GCG AGG AAG CGG GTA AAC GAG TTA CGG GAC  
CTC ATC CGC TAC CAC AAC TAC CGC TAC TAC GTC CTG GCG GAC CCG  
GAG ATC TCC GAC GCC GAG TAC GAC CGG CTT CTT AGG GAG CTC AAG  
GAG CTT GAG GAG CGC TTC CCC GAG CTC AAA AGC CCG GAC TCC CCC  
ACC CTT CAG GTG GGG GCG AGG OCT TTG GAG GCC ACC TTC CGC CCC  
GTC CGC CAC CCC ACC CGC ATG TAC TCC TTG GAC AAC GCC TTT AAC  
CTT GAC GAG CTC AAG GCC TTT GAG GAG CGG ATA GAA CGG GOC CTG  
GGG CGG AAG GGC CCC TTC GOC TAC ACC GTG GAG CAC AAG GTG GAC  
GGG CTT TCC GTG AAC CTC TAC TAC GAG GAG GGG GTC CTG GTC TAC  
GGG GOC ACC GCC GGG GAC GGG GAG GTG GGG GAG GAG GTC ACC CAG  
AAC CTC CTC ACC ATC CCC ACC ATC CCG AGG AGG CTC AAG GGG GTG  
CCG GAG CGC CTC GAG GTC CGG GGG GAG GTC TAC ATG CCC ATA GAG  
GCC TTC CTC CGG CTC AAC GAG GAG CTG GAG GAG CGG GGG GAG AGG  
ATC TTC AAA AAC CCT AGG AAT GCG GCG GCG GGT TCC TTA AGG CAA  
AAA GAC CCC CGC ATC ACC GCC AAG CGG GGC CTC AGG GOC ACC TTC  
TAC GCC TTA GGG CTT GGG CTG GAG GAG GTG GAG AGG GAA GGG GTG  
GCG ACC CAG TTT GOC CTC CTC CAC TGG CTC AAG GAA AAA GGC TTC

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CCC GTG GAG CAC GGC TAC GCC CGG GCC GTG GGG GCG GAA GGG GTG  
 GAG GCG GTC TAC CAG GAC TGG CTC AAG AAG OGG OGG GCG CTT CCC  
 TTT GAG GCG GAC GGG GTG GTG GTG AAG CTG GAC GAG CTT GOC CTT  
 TGG CGG GAG CTC GGC TAC ACC GOC OGC GCC OOC OGG TTC GOC ATC  
 GOC TAC AAG TTC CCC GOC GAG GAG AAG GAG AOC OGG CTT TTG GAC  
 GTG GTC TTC CAG GTG GGG CGC ACC GGG OGG GTG ACC CCC GTG GGG  
 ATC CTC GAG CCC GTC TTC CTA GAG GGC AGC GAG GTC TOC OGG GTC  
 ACC CTG CAC AAC GAG AGC TAC ATA GAG GAG TTG GAC ATC OGC ATC  
 GGG GAC TGG GTT TTG GTG CAC AAG GCG GGC GGG GTC ATC CCC GAG  
 GTC CTC CGG GTC CTC AAG GAG AGG OGC ACG GGG GAG GAA AGG CCC  
 ATT OGC TGG CCC GAG ACC TGC CCC GAG TGC GGC CAC OGC CTC CTC  
 AAG GAG GGG AAG GTC CAC CGC TGC CCC AAC CCC TTG TGC CCC GCC  
 AAG OGC TTT GAG GCC ATC OGC CAC TTC GCC TOC CGC AAG GOC ATG  
 GAC ATC CAG GGC CTG GGG GAA AAG CTC ATT GAG AGG CTT TTG GAA  
 AAG GGG CTG GTC AAG GAC GTG GOC GAC CTC TAC OGC TTG AGA AAG  
 GAA GAC CTG GTG GGC CTG GAG OGC ATG GGG GAG AAG AGC GCC CAA  
 AAC CTC CTC CGC GAG ATA GAG GAG AGC AAG AAA AGA GGC CTG GAG  
 OGC CTC CTC TAC GCC TTG GGG CTT CCC GGG GTG GGG GAG GTC TTG  
 GCC CGG AAC CTG GCG GCC CGC TTC GGG AAC ATG GAC OGC CTC CTC  
 GAG GOC AGC CTG GAG GAG CTC CTG GAG GTG GAG GAG GTG GGG GAG  
 CTC ACG GCG AGG GCC ATC CTG GAG ACC TTG AAG GAC CCC GOC TTC  
 OGC GAC CTG GTA CGG AGG CTC AAG GAG GCG GGG GTG GAG ATG GAG  
 GCC AAG GAG AAG GGC GGG GAG GOC CTT AAA GGG CTC ACC TOC GTG  
 ATC ACC GGG GAG CTT TCC CGC CCC OGG GAA GAG GTG AAG GOC CTC  
 CTA AGG CGC CTC GGG GCC AAG GTG ACG GAC TOC GTG AGC OGG AAG  
 ACG AGC TAC CTC GTG GTG GGG GAG AAC CCG GGG GAG AAC CCG GGG  
 AGC AAG CTG GAG AAG GCC AGG GOC CTC GGG GTC CCC ACC CTC ACG  
 GAG GAG GAG CTC TAC CGG CTC CTG GAG GCG CGG ACG GGG AAG AAG  
 GCG GAG GAG CTC GTC TAA AGGCTTCC.

5. An expression vector comprising a DNA sequence  
 encoding for a thermostable ligase selected from the group  
 consisting of (1) *Thermus aquaticus* HB8 ligase; (2) a  
 thermostable ligase having at least 6 sequential amino acid

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residues corresponding to 6 sequential amino acid residues in *Thermus aquaticus* HB8 ligase; and (3) a ligase active mutant of *Thermus aquaticus* HB8 ligase or a fragment thereof wherein an amino acid residue has been inserted, substituted or deleted in or from the amino acid sequence of the ligase or its fragment.

6. A purified isolated polypeptide which catalyses the formation of a phosphodiester bond at the site of a single-stranded break in duplex DNA at temperatures of about 50°C to about 85°C, and which does not become irreversibly denatured and lose its catalytic ability when subjected to temperatures of from 90°C to about 105°C.

7. A purified isolated polypeptide having the amino acid sequence:

Met Thr Leu Glu Glu Ala Arg Lys Arg Val Asn Glu Leu Arg Asp  
Leu Ile Arg Tyr His Asn Tyr Arg Tyr Tyr Val Leu Ala Asp Pro  
Glu Ile Ser Asp Ala Glu Tyr Asp Arg Leu Leu Arg Glu Leu Lys  
Glu Leu Glu Glu Arg Phe Pro Glu Leu Lys Ser Pro Asp Ser Pro  
Thr Leu Gln Val Gly Ala Arg Pro Leu Glu Ala Thr Phe Arg Pro  
Val Arg His Pro Thr Arg Met Tyr Ser Leu Asp Asn Ala Phe Asn  
Leu Asp Glu Leu Lys Ala Phe Glu Glu Arg Ile Glu Arg Ala Leu  
Gly Arg Lys Gly Pro Phe Ala Tyr Thr Val Glu His Lys Val Asp  
Gly Leu Ser Val Asn Leu Tyr Tyr Glu Glu Gly Val Leu Val Tyr  
Gly Ala Thr Arg Gly Glu Gly Glu Val Gly Glu Glu Val Thr Gln  
Asn Leu Leu Thr Ile Pro Thr Ile Pro Arg Arg Leu Lys Gly Val  
Pro Glu Arg Leu Glu Val Arg Gly Glu Val Tyr Met Pro Ile Glu  
Ala Phe Leu Arg Leu Asn Glu Glu Leu Glu Glu Arg Gly Glu Arg  
Ile Phe Lys Asn Pro Arg Asn Ala Ala Ala Gly Ser Leu Arg Gln  
Lys Asp Pro Arg Ile Thr Ala Lys Arg Gly Leu Arg Ala Thr Phe  
Tyr Ala Leu Gly Leu Gly Leu Glu Glu Val Glu Arg Glu Gly Val  
Ala Thr Gln Phe Ala Leu Leu His Trp Leu Lys Glu Lys Gly Phe

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Pro Val Glu His Gly Tyr Ala Arg Ala Val Gly Ala Glu Gly Val  
 Glu Ala Val Tyr Gln Asp Trp Leu Lys Lys Arg Arg Ala Leu Pro  
 Phe Glu Ala Asp Gly Val Val Val Lys Leu Asp Glu Leu Ala Leu  
 Try Arg Glu Leu Gly Tyr Thr Ala Arg Ala Pro Arg Phe Ala Ile  
 Ala Tyr Lys Phe Pro Ala Glu Glu Lys Glu Thr Arg Leu Leu Asp  
 Val Val Phe Gln Val Gly Arg Thr Gly Arg Val Thr Pro Val Gly  
 Ile Leu Glu Pro Val Phe Leu Glu Gly Ser Glu Val Ser Arg Val  
 Thr Leu His Asn Glu Ser Tyr Ile Glu Glu Leu Asp Ile Arg Ile  
 Gly Asp Trp Val Leu Val His Lys Ala Gly Gly Val Ile Pro Glu  
 Val Leu Arg Val Leu Lys Glu Arg Arg Thr Gly Glu Glu Arg Pro  
 Ile Arg Trp Pro Glu Thr Cys Pro Glu Cys Gly His Arg Leu Leu  
 Lys Glu Gly Lys Val His Arg Cys Pro Asn Pro Leu Cys Pro Ala  
 Lys Arg Phe Glu Ala Ile Arg His Phe Ala Ser Arg Lys Ala Met  
 Asp Ile Gln Gly Leu Gly Glu Lys Leu Ile Glu Arg Leu Leu Glu  
 Lys Gly Leu Val Lys Asp Val Ala Asp Leu Tyr Arg Leu Arg Lys  
 Glu Asp Leu Val Gly Leu Glu Arg Met Gly Glu Lys Ser Ala Gln  
 Asn Leu Leu Arg Gln Ile Glu Glu Ser Lys Lys Arg Gly Leu Glu  
 Arg Leu Leu Tyr Ala Leu Gly Leu Pro Gly Val Gly Glu Val Leu  
 Ala Arg Asn Leu Ala Ala Arg Phe Gly Asn Met Asp Arg Leu Leu  
 Glu Ala Ser Leu Glu Glu Leu Leu Glu Val Glu Glu Val Gly Glu  
 Leu Thr Ala Arg Ala Ile Leu Glu Thr Leu Lys Asp Pro Ala Phe  
 Arg Asp Leu Val Arg Arg Leu Lys Glu Ala Gly Val Glu Met Glu  
 Ala Lys Glu Lys Gly Gly Glu Ala Leu Lys Gly Leu Thr Phe Val  
 Ile Thr Gly Glu Leu Ser Arg Pro Arg Glu Glu Val Lys Ala Leu  
 Leu Arg Arg Leu Gly Ala Lys Val Thr Asp Ser Val Ser Arg Lys  
 Thr Ser Tyr Leu Val Val Gly Glu Asn Pro Gly Ser Lys Leu Glu  
 Lys Ala Arg Ala Leu Gly Val Pro Thr Leu Thr Glu Glu Glu Leu  
 Tyr Arg Leu Leu Glu Ala Arg Thr Gly Lys Lys Ala Glu Glu Leu  
 Val

8. A purified polypeptide isolated from a recombinant organism transformed with a vector that codes for the expression of *Thermus aquaticus* ligase which polypeptide catalyses the formation of a phosphodiester bond at the site of a single-

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stranded break in duplex DNA at a temperature of about 50°C to about 85°C, and which polypeptide does not become irreversibly denatured and lose its catalytic ability when subjected to temperatures in the range of from about 90°C to about 105°C.

9. A purified isolated ligase which catalyses the formation of a phosphodiester bond between two adjacent oligonucleotides hybridized to a complementary strand of DNA at a temperature in the range of about 50°C to about 85°C.

10. A purified isolated ligase which catalyses ligation of two adjacent oligonucleotides hybridized to a complementary target sequence of DNA at a temperature in the range of about 50°C to about 85°C, wherein the products formed of the ligation are about 50 to about 500 more than if a single base mismatch is present at the junction of the adjacent oligonucleotides.

11. A purified isolated ligase which retains the ability to catalyze formation of a phosphodiester bond between two adjacent oligonucleotides hybridized to a complementary strand of DNA at a temperature of about 50°C to about 85°C after repeated prior exposure to temperatures of from about 90°C to about 105°C for about 0.25 min to about 4 minutes.

12. A purified isolated ligase which retains the ability to catalyze formation of a phosphodiester bond between two adjacent oligonucleotides hybridized to a complementary strand of DNA at a temperature range of about 50°C to about 85°C and wherein the products formed of the ligation are about 50 to about 500 more than if a single base mismatch is present at the

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junction of the adjacent oligonucleotides, after repeated prior exposures to a temperature range of from about 90°C to about 105°C for about 0.25 min to about 4 minutes.

13. A method for amplifying a nucleic acid test substance of known nucleotide sequence comprising:

(1) providing a reaction mixture comprising a first set of two adjacent oligonucleotides complementary to and in molar excess of a target sequence nucleic acid and further having no mismatch to the target sequence DNA at the junction of the adjacent oligonucleotides;

(2) providing a thermostable ligase which does not become irreversibly denatured and lose its catalytic ability when subjected to temperatures of from about 50°C to about 105°C; and

(3) subjecting the ligase-mixture to at least two temperature cycles which comprises a first temperature range of about 90°C to 105°C and a second temperature range of about 50°C to about 85°C.

14. A method for detecting a biologically derived nucleic acid test substance having a known normal nucleotide sequence and a known possible mutation at at least one target nucleotide position in the sequence which comprises:

(1) providing a reaction mixture comprising two adjacent oligonucleotides complementary to the target sequence nucleic acid and wherein the oligonucleotides have at least one mismatched base pair to the mutant target

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sequence nucleic acid but not to the normal nucleotide sequence nucleic acid at the junction of the adjacent nucleotides;

(2) providing a second reaction mixture comprising two adjacent oligonucleotides complementary to the target sequence nucleic acid and having at least one mismatched base pair to the normal target sequence nucleic acid, but no mismatch to the mutant target sequence nucleic acid at the junction of the adjacent oligonucleotides;

(3) providing a thermostable ligase which does not become irreversibly denatured and lose catalytic activity when subjected to temperatures of from 50°C to about 105°C to each of the first and second reaction mixtures;

(4) subjecting each of the ligase-mixture to at least one temperature cycle which comprises a first temperature of from about 90°C to about 105°C and a second temperature of about 50°C to about 85°C;

(5) allowing the adjacent oligonucleotides in each reaction mixture to become possibly covalently linked;

(6) separating the test substance and unlinked oligonucleotides in each reaction mixture from possible covalently linked oligonucleotides; and

(7) detecting the presence or absence of covalently linked oligonucleotide product in each reaction mixture wherein the presence of covalently linked oligonucleotides in the first reaction mixture indicates the presence of normal sequence



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and the presence of covalently linked oligonucleotides in the second mixture indicates the presence of mutant sequences.

15. A kit for assaying a biologically derived DNA or RNA test substance which has a known normal nucleotide sequence and a known possible mutation at at least one target nucleotide position in the sequence, the kit comprising:

(1) a container holding a first reaction mixture comprising two adjacent oligonucleotides complementary to the target sequence nucleic acid, wherein one oligonucleotide is labelled and there is at least one mismatched base pair to the mutant target sequence nucleic acid, but not to the normal target sequence nucleic acid at the junction of the adjacent oligonucleotides;

(2) a container holding a second reaction mixture comprising two adjacent oligonucleotides complementary to the target sequence nucleic acid, wherein one oligonucleotide is labelled, wherein there is at least one mismatched base pair to the normal target sequence nucleic acid, but not to the mutant target sequence nucleic acid at the junction of the adjacent oligonucleotides; and

(3) a thermostable ligase which does not become irreversibly denatured and lose its catalytic ability when subjected to temperatures of from 50°C to about 105°C.

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FIG. 1

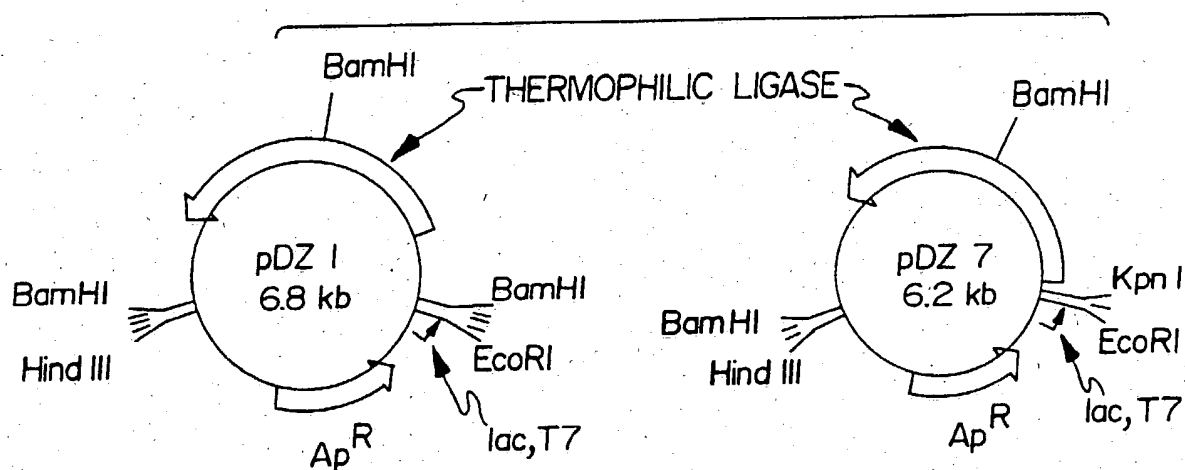
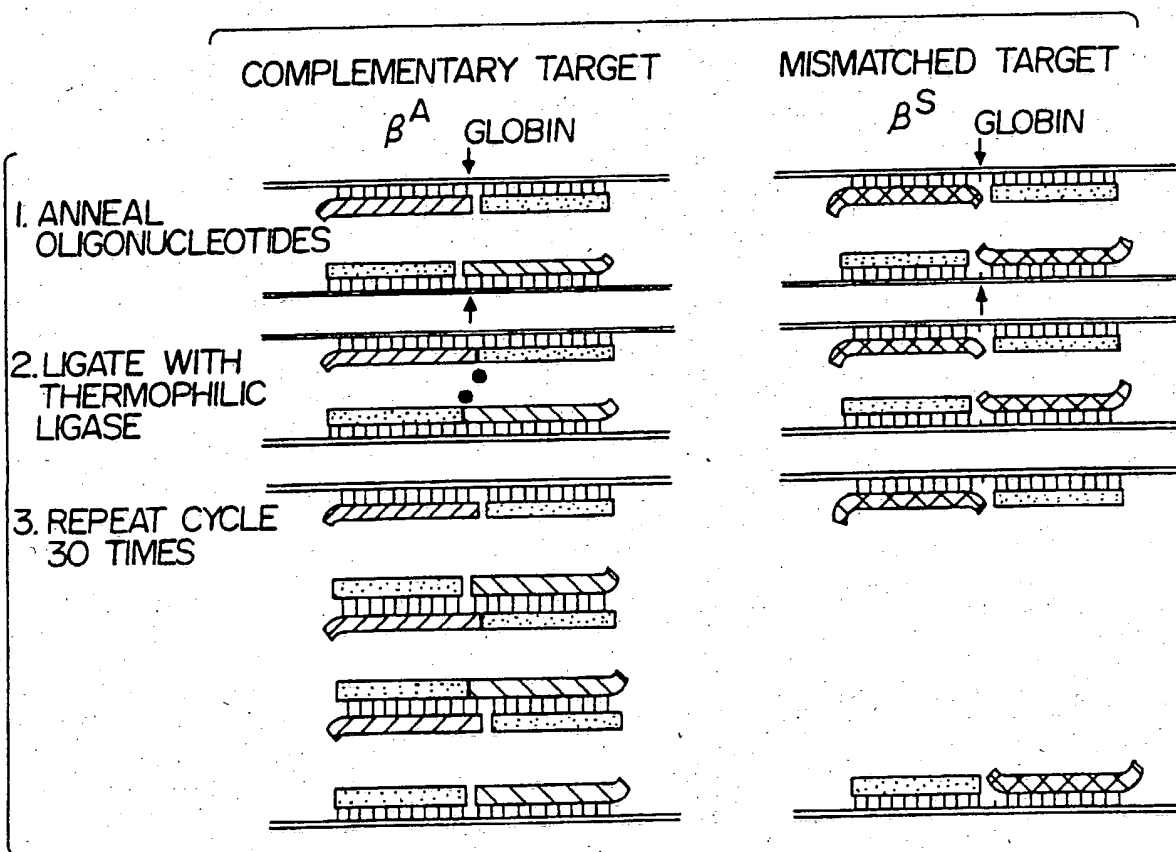


FIG. 2



# LDR Amplification

6x10<sup>6</sup> molecules  
= 10 attomoles

# LDR Amplification

6x10<sup>8</sup> molecules  
= 1 femtomole

Target DNA	101	104	102	105	101	104	102	105	103	106	103	106	101	102	101	102	103	103
Oligonucleotide	101	104	102	105	101	104	102	105	103	106	103	106	101	102	101	102	103	103
Target DNA	B <sup>A</sup>	B <sup>A</sup>	B <sup>S</sup>	B <sup>S</sup>	B <sup>S</sup>	B <sup>S</sup>	B <sup>A</sup>	B <sup>A</sup>	B <sup>A</sup>	B <sup>S</sup>	B <sup>S</sup>	B <sup>S</sup>	B <sup>A</sup>	B <sup>S</sup>	B <sup>S</sup>	B <sup>A</sup>	B <sup>A</sup>	B <sup>S</sup>
Oligo Base	A	T	T	A	A	T	T	A	G	C	G	C	A	T	T	A	G	C
Target Base	T	A	A	T	A	T	T	A	T	A	A	A	T	A	T	A	T	A
CYCLES	5,20																	

47,48 B<sup>S</sup>  
46,45 B<sup>A</sup>

B<sup>S</sup> 48 —  
B<sup>A</sup> 47 —  
B<sup>A</sup> 46 —  
B<sup>A</sup> 45 —

FIG. 3



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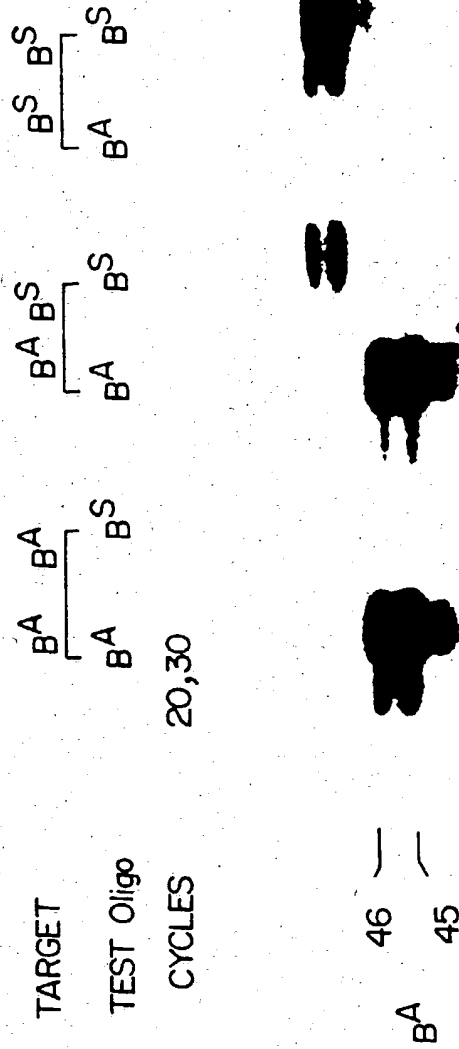
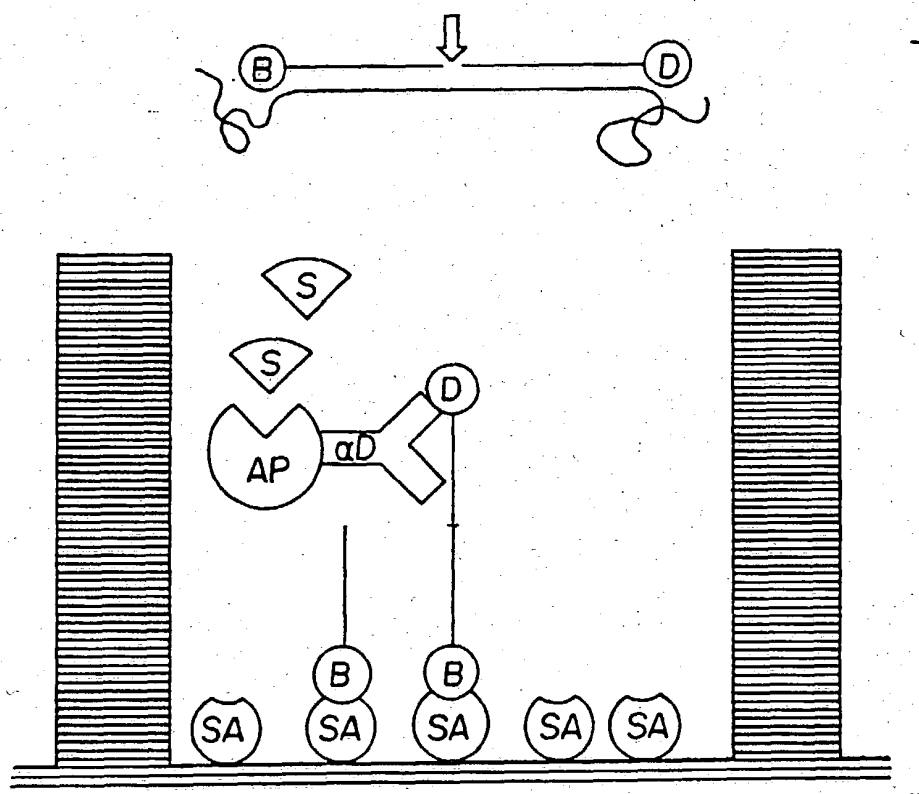


FIG. 5

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FIG. 6



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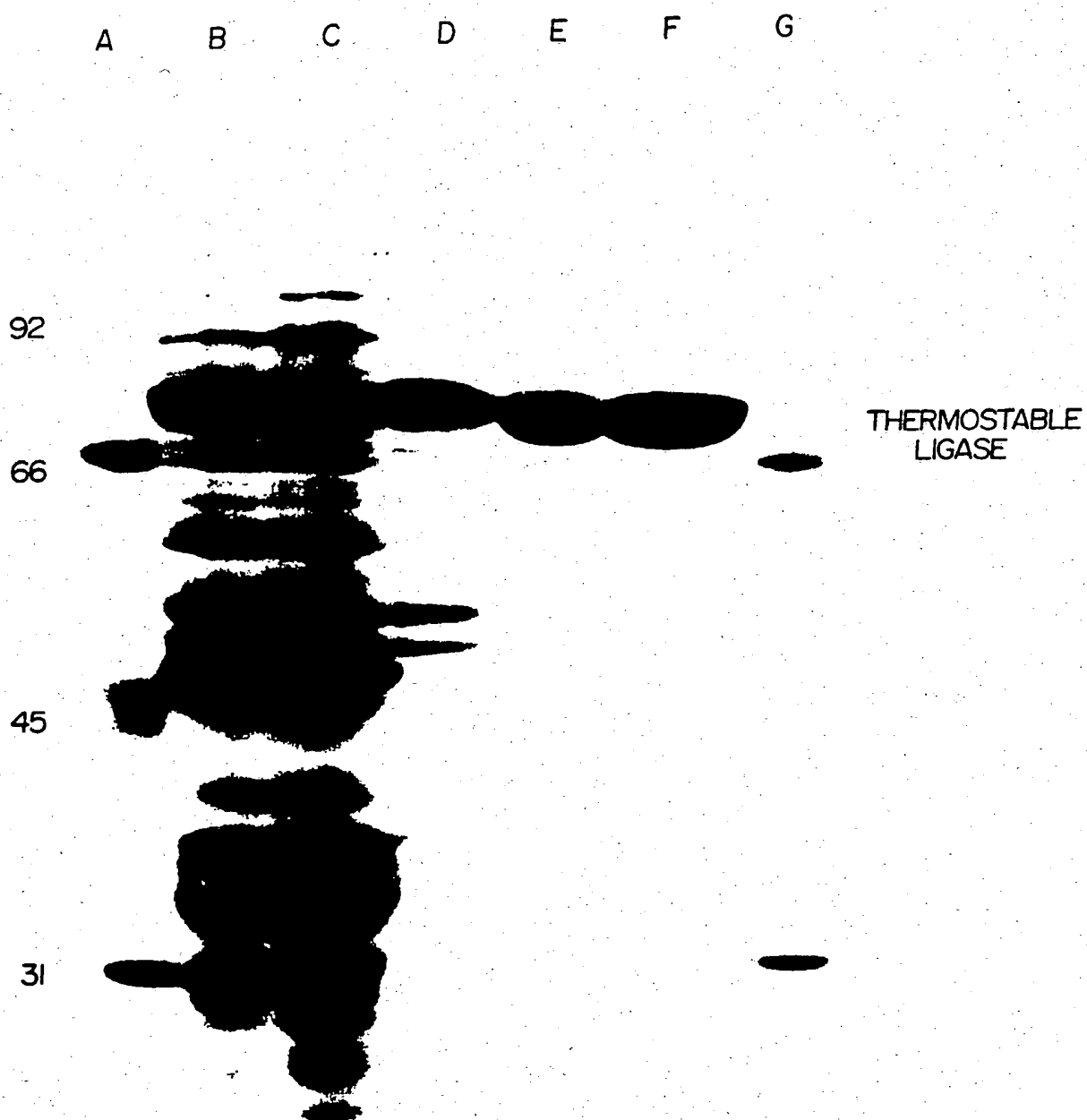
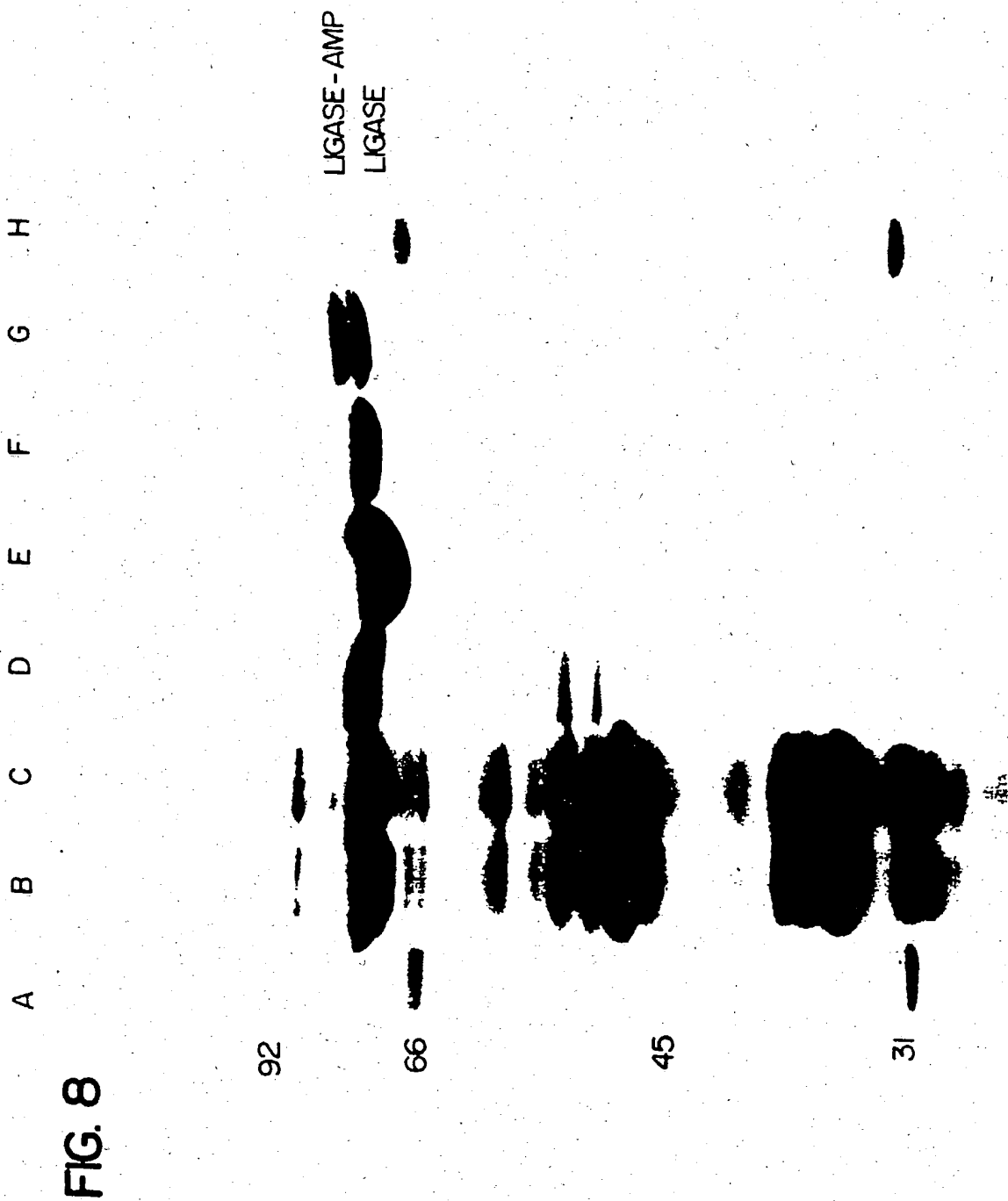


FIG. 7

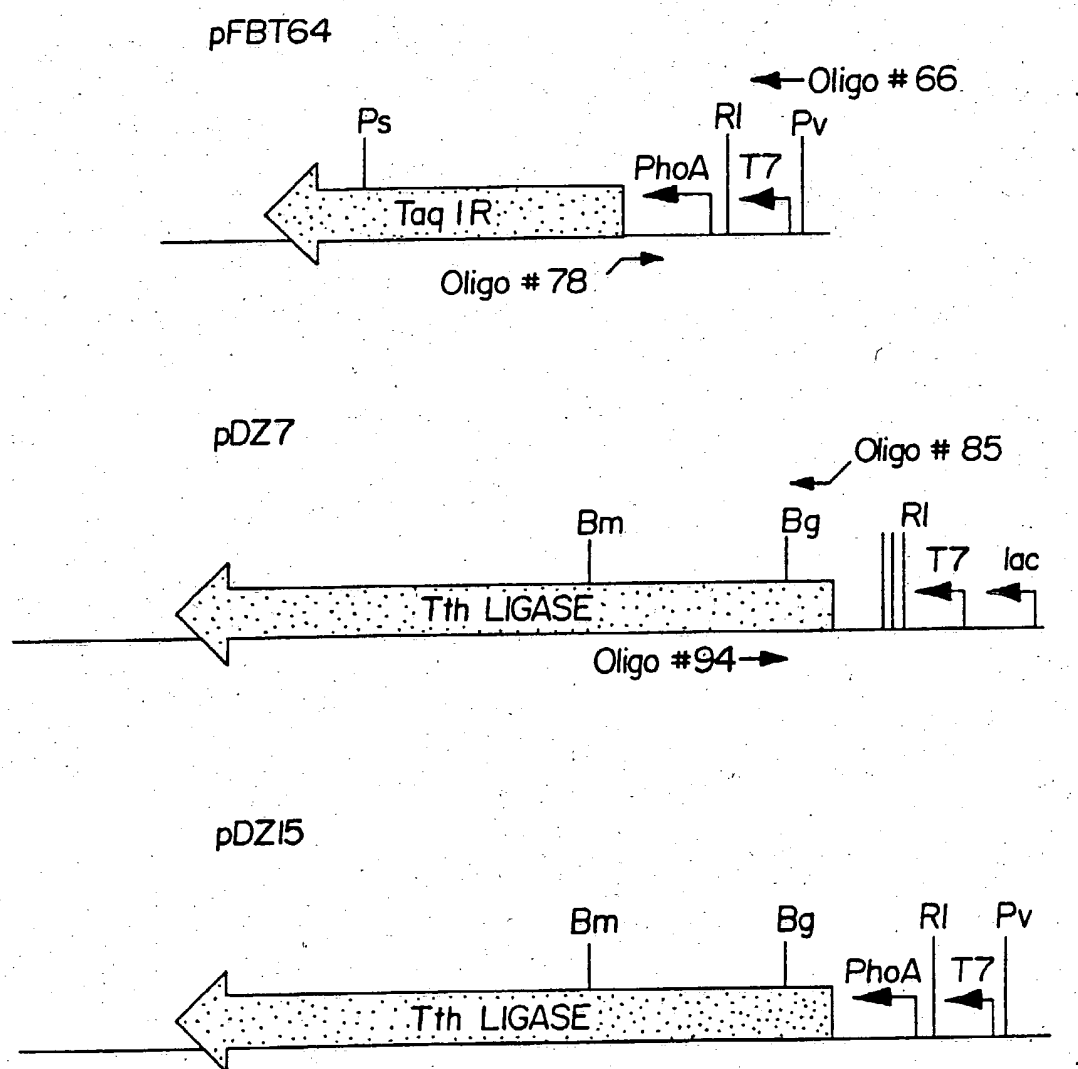
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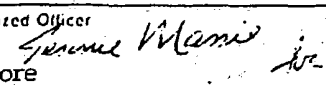
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FIG. 9



# INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/02968

<b>I. CLASSIFICATION OF SUBJECT MATTER</b> (if several classification symbols apply, indicate all) <sup>6</sup> According to International Classification (IPC) or to both National Classification and IPC: <b>IPC(5):</b> C12M 1.03; C12N 9/00, 15/52, 15/70; C12Q 1/25 US Cl: 422/61; 435/183, 252.33, 320.1; 436/63; 536/27; 935/14, 27, 77		
Minimum Documentation Searched <sup>7</sup>		
Classification System	Classification Symbols	
U.S. Cl.	422/61; 435/183; 435/252.33; 435/320.1; 436/63	
Documentation Searched other than Minimum Documentation to the extent that such Documents are included in the Fields Searched <sup>8</sup>		
APS, Chemical Abstracts/STN, Biosis/DIALOG databases		
<b>III. DOCUMENTS CONSIDERED TO BE RELEVANT <sup>9</sup></b>		
Category <sup>*</sup>	Citation of Document, <sup>11</sup> with indication, where appropriate, of the relevant passages <sup>12</sup>	Relevant to Claim No. <sup>13</sup>
Y	Chemical Abstracts, Vol. 79, No. 13, issued 01 October 1973, Konrad et al, "Genetic and enzymic characterization of a conditional lethal mutant of <u>Escherichia coli</u> K12 with a temperature sensitive DNA ligase", 75781v, pages 75781-75782. See entire document.	1-4, 6-12
Y	GENE, Vol. 56, No. 1, issued 1987, Barany, F, "A genetic system for isolation and characterization of <u>TaqI</u> restriction endonuclease mutants", pages 13-27, especially pages 14, 17, and 25.	1-4, 6-12
Y	NUCLEIC ACIDS RESEARCH, Vol. 14, No. 20, issued 24 October 1986, Howard et al, "Cloning the <u>DdeI</u> restriction-modification system using a two-step method", pages 7939-7951, especially pages 7939-7940, 7946-7947, and 7949-7950.	1-4, 6-12
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 264, No. 11, issued 15 April 1989, Lawyer et al, "Isolation, Characterization, and Expression in <u>Escherichia coli</u> of the DNA Polymerase Gene from <u>Thermus aquaticus</u> ", pages 6427-6437, especially pages 6427-28, 6436-38.	3-12
<div style="display: flex; justify-content: space-between;"> <div style="width: 45%;"> <p><sup>*</sup> Special categories of cited documents: <sup>10</sup></p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div style="width: 45%;"> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</p> <p>"&amp;" document member of the same patent family</p> </div> </div>		
<b>IV. CERTIFICATION</b>		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
21 August 1991		29 AUG 1991
International Searching Authority		Signature of Authorized Officer
ISA/US		 William Moore

## III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation	Document, with indication, where appropriate, of the relevant pages	Relevant to Claim No
Y	THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol. 259, No. 16, issued 25 August 1984, Takahashi et al, "Thermophilic DNA Ligase", pages 10041-10047, see entire document.		3-12
Y	SCIENCE, Vol. 241, No. 4869, issued 26 August 1988, Landegren et al, "A Ligase-Mediated Gene Detection Technique", pages 1077-1080, see entire document.		13-14
Y	GENOMICS, Vol. 4, No. 4, issued May 1989, Wu et al, "The Ligation Amplification Reaction (LAR) - Amplification of Specific DNA Sequences Using Sequential Rounds of Template-Dependent Ligation", pages 560-569, see entire document.		13-14
Y	GENE, Vol. 76, No. 2, issued 30 March 1989, Wu et al, "Specificity of the nick-closing activity of bacteriophage T4 DNA ligase", pages 245-254, especially pages 248-254.		14
Y	SCIENCE, Vol. 242, No. 4876, issued 14 October 1988, Landegren et al, "DNA Diagnostics - Molecular Techniques and Automation", pages 229-237, especially pages 235-236.		15
Y	LANDEGREN ET AL, "Automated Gene Detection", Symposium on Biotechnology and Human Genetic Predisposition to Disease, 18th Annual UCLA Symposia on Molecular and Cellular Biology, March 27-April 3, 1989, abstract K 217, page 31.		15
A	GENE, Vol. 89, No. 1, issued 30 April 1990, Barringer et al, "Blunt-end and single strand ligation by <u>Escherichia coli</u> ligase: influence on an <u>in vitro</u> amplification scheme", pages 117-122.		
A,P	JOURNAL OF THE NATIONAL CANCER INSTITUTE, Vol. 82, No. 18, issued 19 September 1990, Resnick et al, "Detection and Typing of Human Papillomavirus in Archival Cervical Cancer Specimens by DNA Amplification With Consensus Primers", pages 1477-1484.		

## FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

A	US 4,889,818 (GELFAND ET AL) 26 DECEMBER 1989
A	US 4,879,214 (KOHNER ET AL) 07 NOVEMBER 1989

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE<sup>1</sup>

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers \_\_\_\_\_, because they relate to subject matter <sup>12</sup> not required to be searched by this Authority, namely:
  
2. ☐ Claim numbers \_\_\_\_\_, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out <sup>11</sup>, specifically:
  
3. ☐ Claim numbers \_\_\_\_\_, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING<sup>2</sup>

This International Searching Authority found multiple inventions in this international application as follows:

**I. Claims 1-13 drawn to a first method of making, a first product, and a first method of use of that product.**

**II. Claims 14-15 drawn to a second product and a method of use of that second product.**

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
  
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
  
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

## Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☒ No protest accompanied the payment of additional search fees.